Dobrava and Tula hantaviruses from Central Europe: molecular evolution and pathogenic relevance

D I S S E R T A T I O N

zur Erlangung des akademischen Grades

d o c t o r r e r u m n a t u r a l i u m

(Dr. rer. nat.)

im Fach Biologie

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät I

der Humboldt-Universität zu Berlin

von

Diplom-Biologe Boris Klempa

geboren am 5. Dezember 1976 in Malacky, Slowakei

Präsident der Humboldt-Universität zu Berlin

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Tag der mündlichen Prüfung: 09. Dezember 2004

CONTENTS

SUMMARY

Hantaviruses are rodent-borne bunyaviruses that cause hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. The viruses form a genus *Hantavirus* within the *Bunyaviridae* family. They are negative-strand RNA viruses with a genome consisting of three different segments, S, M, and L. Hantaviruses belong to the group of "emerging viruses" exhibiting an increasing significance as human pathogens. To cause human disease, the viruses have to be transmitted from their respective hosts to men.

There is an urgent need to acquire substantial knowledge about the epidemiology, molecular evolution and clinical relevance of hantaviruses circulating in Central Europe. This thesis presents novel data about two European hantaviruses, Dobrava virus (DOBV) and Tula virus (TULV).

DOBV is an important etiologic agent of HFRS in Europe. DOBV strains were found to be hosted by at least two different rodent species, yellow-necked mouse (*Apodemus flavicollis*) and striped field mouse (*A. agrarius*). According to their natural hosts they form the distinct genetic lineages DOBV-Af and DOBV-Aa, respectively. We have determined and analysed the complete S and M, and partial L segment nucleotide sequences of sympatrically occurring DOBV-Af and DOBV-Aa strains from Central Europe. Molecular phylogenetic analyses gave evidence for genetic reassortment in the evolution of the virus species. It will be important to see whether such reassortment processes (similar to the situation in influenza viruses which carry segmented genomes, too) can change the virulence of hantaviruses towards humans.

Whereas for virus strains of the DOBV-Af lineage their pathogenic potential towards humans was known from studies on the Balkans, such evidence was still missing for the newly discovered DOBV-Aa lineage in Central Europe. We were able to amplify a DOBV-Aa nucleotide sequence from a DOBV-seropositive HFRS patient in Central Europe. This is the first molecular identification of human infection by DOBV in Central Europe and the first direct proof that a virus strain related to the DOBV-Aa lineage, carried by *A. agrarius* rodents, is able to cause HFRS.

For future studies on the virus-host interactions of DOBV-Aa, it was important to isolate a viable virus strain. This urgency was underlined by the fact that the Central European DOBV-Aa strains were shown to be only distantly related to the existing DOBV cell culture isolates from Estonia, Slovenia and Greece. Therefore, under biosafety level 3 conditions, we have established a DOBV isolate named Slovakia (SK/Aa) from an *A. agrarius* animal captured in Slovakia. SK/Aa, as the only isolate clearly belonging to the

DOBV-Aa lineage, can be taken as the representative of this virus lineage. The new virus isolate, in comparison to a DOBV-Af strain, was used for serotyping neutralising antibodies of HFRS patients in Central Europe by the use of a focus reduction neutralisation assay. Most patients' sera exhibited a higher end-point titer towards SK/Aa suggesting that DOBV-Aa strains are responsible for most of the DOBV HFRS cases in this region.

TULV is carried by European common voles (*Microtus* sp.). Its pathogenic potential for humans was rather unknown. We have described the first case of HFRS which can be associated with TULV infection. Moreover, TULV strains detected in *M. arvalis* near the home village of the patient in North-East Germany clustered with strains from Poland and represent a new, well-supported genetic lineage within the TULV species. In addition to DOBV and longer known Puumala virus, TULV is most likely an additional causative agent of HFRS in Central Europe.

ZUSAMMENFASSUNG

Hantaviren sind Erreger, die von Nagetieren auf den Menschen übertragen werden. In Europa und Asien vorkommende Hantaviren lösen Hämorrhagische Fieber mit Renalem Syndrom (HFRS) aus, in den Amerikas zirkulierende Viren das Hantavirus Cardiopulmonale Syndrom. Die Viren bilden ein eigenes Genus *Hantavirus* innerhalb der Familie *Bunyaviridae*. Sie sind Negativstrang-RNA Viren, deren Genom aus drei Segmenten (S, M, L) besteht. Hantaviren gehören zur Gruppe der „emerging viruses“, die durch eine zunehmende Bedeutung als allgemeingefährliche Humanpathogene gekennzeichnet sind. Die einzelnen Virusspecies sind mit unterschiedlichen Nager-Wirtsspecies assoziiert und werden von diesen auf den Menschen übertragen, der einen Fehlwirt darstellt.

Es besteht die dringende Notwendigkeit, neue, grundsätzliche Erkenntnisse zur Epidemiologie, molekularen Evolution und klinischen Relevanz der in Mitteleuropa zirkulierenden Hantaviren zu gewinnen. Die vorgelegte Arbeit beinhaltet derartige Ergebnisse zu zwei europäischen Hantaviren, dem Dobravavirus (DOBV) und dem Tulavirus (TULV).

DOBV ist ein wichtiger HFRS-Erreger in Europa. DOBV Stämme kommen in mindestens zwei verschiedenen Nagerspecies, der Gelbhalsmaus (*Apodemus flavicollis*) und der Brandmaus (*Apodemus agrarius*) vor. In Übereinstimmung mit diesen unterschiedlichen natürlichen Wirten konnten wir zeigen, dass die Virusstämme zwei genetische Linien innerhalb der DOBV Species bilden: DOBV-Af und DOBV-Aa. Es wurden die vollständigen Nukleotidsequenzen der S- und M-Segmente sowie partielle Sequenzen der L-Segmente von sympatrisch vorkommenden DOBV-Af und DOBV-Aa Stämmen aus Mitteleuropa bestimmt und für molekularphylogenetische Analysen genutzt. Die Analysen zeigten das Vorkommen von Reassortmentprozessen der Genomsegmente während der Evolution der Virusspecies. Für Influenzaviren (die ebenfalls segmentierte Genome besitzen) ist bekannt, dass Reassortment die wichtigste genetische Grundlage für Veränderungen der Pathogenität der Viren ist. Unsere Ergebnisse schaffen nun die Voraussetzung, diese Prozesse ebenfalls für die Hantaviren zu untersuchen.

Während für Infektionen mit Virusstämmen der DOBV-Af Linie in Südosteuropa das Auftreten von mittelschwerem und schwerem HFRS beschrieben wurde, gab es bisher keinen Beweis für die Humanpathogenität der neu entdeckten DOBV-Aa Stämme. Es konnte nun die virale Nukleotidsequenz aus einem DOBV-seropositiven HFRS-Patienten in Mitteleuropa amplifiziert und analysiert werden. Damit wurde erstmalig der molekulare Beweis erbracht, dass DOBV in Mitteleuropa HFRS auslöst und dass die DOBV-Aa Linie aus *Apodemus agrarius* humanpathogen ist.

Für weitere Forschungen zu den Virus-Wirt-Interaktionen von DOBV-Aa ist das Vorhandensein eines vermehrungsfähigen DOBV-Aa Virusisolates die entscheidende Voraussetzung. Diese Notwendigkeit wird durch die Tatsache unterstrichen, dass die mitteleuropäischen DOBV-Aa Stämme mit den existierenden DOBV Isolaten aus Slowenien, Griechenland und Estland nur relativ entfernt verwandt sind. Unter Laborbedingungen der Sicherheitsstufe 3 wurde aus einer in der Slowakei gefangenen *Apodemus agrarius* Maus ein neues Virusisolat gewonnen, welches „Slovakia (SK/Aa)“ genannt wurde. SK/Aa ist das bisher einzige Virusisolat, das die DOBV-Aa Linie repräsentiert. Es wurde gemeinsam mit einem Isolat der DOBV-Af Linie zur vergleichenden Typisierung der Antikörper von mitteleuropäischen HFRS-Patienten mittels Fokusreduktionsneutralisationstest eingesetzt. Die Seren der meisten Patienten zeigten die höchsten neutralisierenden Antikörpertiter gegenüber SK/Aa, was die Schlussfolgerung zulässt, dass DOBV-Aa Stämme für die meisten DOBV-Infektionen in Mitteleuropa verantwortlich sind.

TULV wird durch die Feldmaus (*Microtus arvalis*) beherbergt. Die Übertragbarkeit auf den Menschen bzw. die Fähigkeit zur Auslösung von HFRS waren bisher wenig bzw. nicht bekannt. Wir haben den ersten Fall von HFRS gefunden, der mit einer TULV Infektion assoziiert ist. Aus demselben geographischen Gebiet in Nordostdeutschland, in dem dieser Patient lebt, konnten aus Feldmäusen TULV Nukleotidsequenzen amplifiziert werden. In phylogenetischen Analysen clustern sie mit Stämmen aus Polen und bilden mit diesen gemeinsam eine eigene, neue genetische Linie innerhalb der TULV Species. Ausser dem hier untersuchten DOBV und dem länger bekannten Puumalavirus ist TULV offenbar das dritte Hantavirus, das in Mitteleuropa HFRS hervorruft.

1. INTRODUCTION

Hantaviruses represent a unique genus *Hantavirus* within the *Bunyaviridae* family. They are ”emerging viruses” which cause two human zoonoses, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) (Krüger *et al*., 2001; Mertz *et al*., 1997; Plyusnin *et al*., 2001a; Schmaljohn and Hjelle, 1997; Schmaljohn and Nichol, 2001). Prominent examples of hantaviruses that cause human disease are the Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV) and Dobrava virus (DOBV) causing HFRS in Eurasia, and Sin Nombre virus (SNV) and Andes virus (ANDV) causing HCPS in the Americas. In contrast to other genera of the *Bunyaviridae* family, hantaviruses are not transmitted by arthropods. They are spread by aerosolised rodent excreta and produce chronic infection with no apparent harm in their natural hosts, rodents of the family *Muridae.*

1.1 Short historical overview

Human disease now known as HFRS was probably for the first time described in Russian clinical records from Far Eastern Siberia in 1913 (Casals *et al*., 1970). However, Chinese writings from the 10th century already described a disease resembling HFRS (Lee *et al*., 1982). The first steps in elucidation of the etiology, epidemiology and ecology of HFRS were mostly done by military researches as the first epidemics in 20th century often occurred during military conflicts. Military physicians encountered the disease during World War I (“War nephritis”), invasion of Japan to Manchuria, World War II, as well as during the Korean Conflict in 1951 (Korean hemorrhagic fever). Despite a massive effort to isolate the etiologic agent during and after the Korean War, it was not until 1976 that the virus was discovered; the first hantavirus described, named Hantaan virus after the nearby Hantaan river in Korea, was isolated from the striped field mouse, *Apodemus agrarius*, by Ho-Wang Lee and co-workers (Lee *et al*., 1978). Soon, other HFRS-associated viruses such as PUUV from bank voles (Brummer-Korvenkontio *et al*., 1980), and SEOV from urban rats (Lee *et al*., 1982) were isolated using a similar approach. Antibodies to hantaviruses were found in rodents and humans all over the world and new related viruses were later isolated, e.g. Prospect Hill (PHV) in the USA from meadow voles, *Microtus pennsylvanicus*, or DOBV from *A. flavicollis* in Slovenia. Interestingly, the Thottapalyam virus, isolated from the shrew, an insectivore, in

1971 in India (Carey *et al*., 1971) was actually the first hantavirus ever isolated, but its relation to HFRS-causing viruses was detected many years later (Zeller *et al*., 1989; Xiao *et al*., 1994).

A new chapter in hantavirus research was opened in May 1993 when a sudden outbreak of a mysterious influenza-like prodrome of fever and myalgia that evolved into a relentless and often fatal syndrome of shock and edema occurred in the Four Corners region of USA. SNV was discovered as a causative agent of this disease, today known as HCPS (Nichol *et al*., 1993). This virus was shown to be carried by the common deer mouse (*Peromyscus maniculatus*) and other related, so called "New World hantaviruses" were soon discovered in other American rodents.

1.2 Hantaviruses within the *Bunyaviridae* family

Hantaviruses form a separate genus in the *Bunyaviridae* family. The family *Bunyaviridae* contains five genera: *Bunyavirus, Phlebovirus, Nairovirus, Hantavirus,* and *Tospovirus* (Table 1). It represents one of the largest viral families with over 300 viruses. The features that encompass these diverse viruses within a single family are a common morphology, the tripartite genome, the absence of matrix in virions, and budding into intracytoplasmic vesicles from the internal membranes of the Golgi apparatus during replication. Ranging from 80 to 120 nm in size the viral particles are spherical or pleomorphic. They are enveloped viruses with a single stranded RNA genome of mostly negative polarity that is divided into three segments (Pringle, 1991).

Molecular genomic features are used to define the *Bunyaviridae* genera and antigenic data are used to separate the viruses within each genus. The most important human pathogens in the genus *Bunyavirus* include mosquito-borne California serogroup causing acute febrile illnesses and CNS infections (La Crosse, Tahyna). The Bunyamwera serogroup contains mostly tropical arboviruses. Crimean-Congo hemorrhagic fever virus is a representative of the *Nairovirus* genus, distinguished by the fact that its members are all transmitted by ticks. The *Phlebovirus* genus is divided into two groups: the Phlebovirus group with Sandfly fever and Toscana viruses transmitted by *Phlebotomidae* mites and causing a febrile illness with rash, and the Rift Valley fever virus, transmitted by mosquitoes, and causing veterinary and human outbreaks. Tomato spotted wilt virus is the only one member of the genus *Tospovirus* and is transmitted between plants by thrips, plant-feeding arthropods (Beaty and Calisher, 1991).

Hantaviruses (Table 2) are exceptional in that they have no arthropod vector. The

reservoir hosts are specific rodents/insectivores. These features make it of special interest to study the genetic evolution of hantaviruses; the close phylogenetic correspondence between hantaviruses and their reservoir hosts has been interpreted as evidence for coevolution (cospeciation) of virus and host. Hantaviruses can cause severe, life threatening diseases in humans, however, they cause no detectable cytopathology in cell cultures and produce persistent non-pathogenic infections in rodents (Plyusnin *et al*., 1996; Plyusnin and Morzunov, 2001).

Table 1: Family *Bunyaviridae*. Taxonomical classification according to 7th report of The International Committee on Taxonomy of Viruses (ICTV) (Elliott *et al*., 1999) and selected significant pathogens

Genus	Type Species	Host	Selected significant human/veterinary pathogens
Bunyavirus	Bunyamwera virus	Vertebrates	La Crosse virus
			Tahyna virus
			Akabane virus
			Oropouche virus
Hantavirus	Hantaan virus	Vertebrates	Hantaan virus
			Sin Nombre virus
Nairovirus	Dugbe virus	Vertebrates	Crimean-Congo hemorrhagic fever virus
			Nairobi sheep disease virus
Phlebovirus	Rift Valley fever virus	Vertebrates	Rift Valley fever virus
			Sandly fever-Sicilian virus
Tospovirus	Tomato spotted wilt virus	Plants	Tomato spotted wilt virus

Table 2: Species in the genus *Hantavirus*, according to their rodent host.

species*	rodent host	assigned abbreviation
***Murinae*-associated**		
Dobrava-Belgrade virus	*Apodemus flavicollis*	(DOBV)
Hantaan virus	*Apodemus agrarius*	(HTNV)
Seoul virus	*Rattus norvegicus*	(SEOV)
Thailand virus	*Bandicota indica*	(THAIV)
***Arvicolinae*-associated**		
Isla Vista virus	*Microtus califonicus*	(ISLAV)
Khabarovsk virus	*Microtus fortis*	(KHAV)
Prospect Hill virus	*Microtus pennsylvanicus*	(PHV)
Puumala virus	*Clethrionomys glareolus*	(PUUV)
	C. rufocanus	
Topografov virus	*Lemmus sibiricus*	(TOPV)
Tula virus	*Microtus arvalis*	(TULV)
	M. rossiaemeridionalis	
***Sigmodontinae*-associated**		
Andes virus	*Oligoryzomys longicaudatus*	(ANDV)
Bayou virus	*Oryzomys palustris*	(BAYV)
Black Creek Canal virus	*Sigmodon hispidus*	(BCCV)
Cano Delgadito virus	*Sigmodon alstoni*	(CADV)
El Moro Canyon virus	*Reithrodontomys megalotis*	(ELMCV)
Laguna Negra virus	*Calomys laucha*	(LANV)
Muleshoe virus	*Sigmodon hispidus*	(MULV)
New York virus	*Peromyscus leucopus*	(NYV)
Rio Mamore virus	*Oligoyomys microtis*	(RIOMV)
Rio Segundo virus	*Reithrodontomys mexicanus*	(RIOS)
Sin Nombre virus	*Peromyscus maniculatus*	(SNV)
***Insectivores*-associated**		
Thottapalayam virus	*Suncus murinus*	(TPMV)

* Only official virus species are shown. Tentative virus species, strains, or serotypes according to 7th report of ICTV (Elliott *et al*., 1999) are not shown.

1.3 Genome structure and replication

The virus genome consists of three segments of negative-stranded RNA; the large (L) segment encodes the viral RNA polymerase, the medium (M) segment the glycoproteins precursor (GPC), and the small (S) segment the nucleocapsid (N) protein (Figures 1, 2). The viral RNA-dependent RNA polymerase (L protein) acts as a replicase, transcriptase, endonuclease and possibly, RNA helicase. The GPC is cotranslationally cleaved into G1 and G2 proteins, which are thought to form a heterodimer. An open reading frame (ORF) of putative non-structural protein NS_s have been found in many hantaviruses associated with *Arvicolinae* (e.g. PUUV, PHV, TULV) or *Sigmodontinae* rodents (e.g. SNV, BCCV, ELMCV) but not in *Murinae*-associated hantaviruses (HTNV, SEOV, DOBV). Despite the presence of nonstructural proteins in other bunyaviruses, NS_s has not been found in hantavirus-infected cells. The functionality of these ORFs as well as their absence in *Murinae*-associated hantaviruses remains to be explained.

Sequence comparisons of distinct hantavirus species showed 60-70% identity at the nucleotide level for all three RNA segments. The corresponding values for deduced proteins are: 70-90% for L, 60-85% for N and 50-80% for G1, G2 proteins. The length of S segment varies significantly, mostly in its 3' non-coding region (3' NCR). However, within the hantavirus species, the length and sequence of 3' NCR are conserved suggesting a functional role. Supposing the participation of S segment 3' NCR in nucleic acid packaging, this step could be host-specific. In addition, the secondary structure of 3' NCR might be crucial for this step of viral reproduction.

The 5' and 3'-termini of all three genome segments are genus specific, highly conserved and complementary to each other. This enables to form panhandle structures of the RNA segments, which are typical also for other bunyaviruses. They are thought to play a role in viral transcription and in the proposed prime-and-realign mechanism of replication. Panhandles in hantaviruses are at least 17 nt long and the complementarity of the termini is incomplete.

Shortly after virion entry and uncoating in the cytoplasm of host cells, primary transcription by L protein occurs. The signal for switching from primary transcription to replication is most likely the accumulation of N protein as it is well documented for other negative-strand viruses. Transcription initiation requires suitable primers, which are acquired by the L protein from capped cellular mRNAs in the cytoplasm. The "prime-and-realign" model suggests that the terminal G residue of the host-derived primer aligns with the third nucleotide of the hantaviral RNA template (C residue) to initiate the transcription. After synthesis of a few nucleotides, the nascent RNA realigns by slipping backward three

nucleotides on the repeated terminal sequences (AUCAUCAUC) of the L, M, and S RNA segments (Plyusnin *et al*., 1996; Schmaljohn and Nichol, 2001).

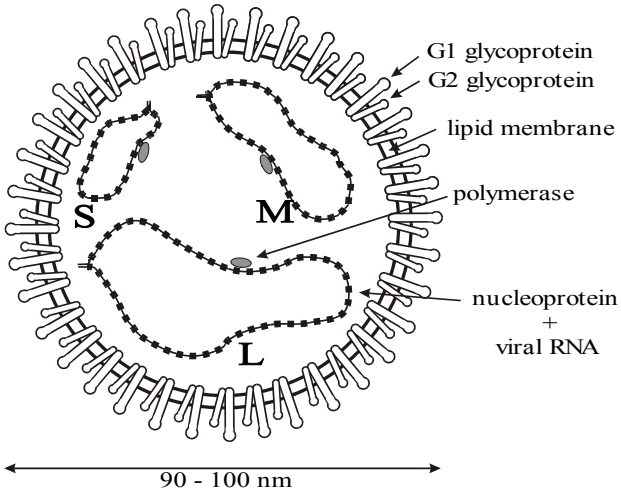

Figure 1: Scheme of a hantavirus particle (own drawing).

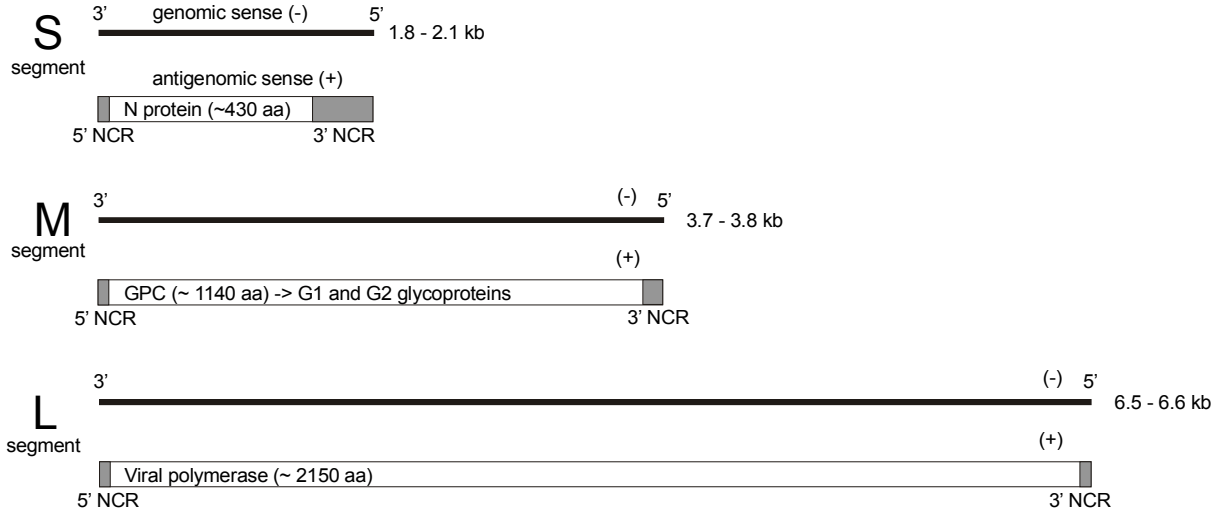

Figure 2: Hantavirus genome structure (adapted from Plyusnin *et al*., 1996).

1.4 Pathogenesis

1.4.1 Infection in natural host *vs.* humans

In both rodents and humans, replication occurs predominantly in pulmonary endothelial cells and macrophages. However, viral antigen is present in many organs and prominent in the spleen, kidney and lung. Hantavirus infection of rodents is asymptomatic. Although neutralising antibodies appear in rodents soon after infection, hantaviruses are not cleared from their rodent hosts. Animals are persistently infected and capable of transmitting the virus. Patients similarly develop neutralising antibody response but are able to clear the virus, suggesting that hantaviruses modulate rodent cellular host responses to effect viral persistence (Mackow and Gavrilovskaya, 2001). Recently, it was shown that HTNV can productively infect dendritic cells, upregulates costimulatory major histocompatibility complex (MHC) and adhesion molecules and induces the release of proinflammatory cytokines. This supports the hypothesis that in humans, hantaviruses elicit a strong immune response which could be an essential part of the virus-associated pathogenesis (Raftery *et al*., 2002).

1.4.2 Human diseases

Hantaviruses are known to cause two human diseases, HFRS and HCPS. They are transmitted to humans from rodents through inhalation of aerosolised excreted virus. Single report from South America suggests also person-to-person transmission of HPS-associated Andes virus (ANDV) but the relevance of this single event remains unclear (Padula *et al*., 1998).

Many clinical symptoms of both HFRS and HCPS are due to increased capillary permeability, which explains the hemorrhagic tendency and abdominal pain due to retroperitoneal edema in HFRS and extravasation of fluid to alveolar space and pulmonary edema occurring in HCPS. The specific feature that enables a certain hantavirus species to cause preferentially renal vs. pulmonary symptoms, or subclinical/mild vs. lethal manifestations remains to be explained (Krüger *et al*., 2001).

1.4.3 Hemorrhagic fever with renal syndrome

The clinical course can be usually divided into five distinct phases. After an incubation period of 2-4 weeks, there is an abrupt onset of disease with fever, chills, general malaise, headache and other influenza-like symptoms, nausea, back and abdominal pain, gastrointestinal symptoms. This febrile phase usually lasts for 3-7 days. Towards the end of this phase, conjunctival hemorrhages and fine petechiae occur at the body surface. The hypotensive phase can last from several hours to two days. The characteristic drop of the

blood platelet begins. In severe cases, a clinical shock state occurs and one third of HFRS deaths are associated with irreversible shock at this stage. In the oliguric phase (3-7 days) due to renal failure, a massive proteinuria occurs. One half of fatalities occur during this phase. Typical findings are elevated concentrations of serum creatinine and urea. Blood pressure becomes normalised or even changes to hypertension. The onset of the diuretic phase is a positive prognostic sign for the patient. Diuresis of 3-6 litres is usually observed. The convalescent phase is characterised by recovery of the clinical and biochemical markers (Krüger *et al*., 2001).

The most severe forms of HFRS are caused by HTNV in Eastern Russia, China and Korea. Severe HFRS cases occur also in Europe, mostly in Balkan region, caused by DOBV. Most clinical cases due to SEOV infection exhibit a milder course than the HTNV infections. PUUV usually causes a rather mild form of HFRS, called *Nephropathia epidemica* (Krüger *et al*., 2001).

1.4.4 Hantavirus cardiopulmonary syndrome

In contrast to the five phases classically known for HFRS, clinical features of HCPS were originally divided into four phases (febrile, cardiopulmonary, diuretic, and convalescent). Febrile phase, typically lasting 3-5 days is characterised by fever, myalgia, and malaise. Other symptoms such as headache, dizziness, anorexia, nausea, vomiting, diarrhoea and abdominal pain may be present. Early recognition of HPS in this period is difficult, and the disease is indistinguishable from other viral prodromes. At the end, announcing the onset of pulmonary edema, non-productive cough and tachypnea occur. Cardiopulmonary phase is characterised by the presentation of shock and pulmonary edema. Hypotension and oliguria can be accompanied by shock. Tachypnea, exertional dyspnea, and non-productive cough are the clinical expression of pulmonary edema. Once pulmonary edema is present, the disease proceeds fast. Patients can decease within 24-48h; hypoxia, circulatory compromise, or both being the immediate cause of death. In diuretic phase, rapid clearance of pulmonary edema as well as resolution of fever and shock occur. Spontaneous diuresis is an early sign of this process. Convalescent phase may last up to two months, with patients recovering apparently completely. However, continued follow up is necessary to determine the long-term persistence of pulmonary dysfunction.

1.5 Virus ecology

Hantaviruses are exclusively maintained in the populations of their specific rodent hosts. Unlike other bunyaviruses, they are not transmitted by arthropod vectors. The order *Rodentia* contains the most species of mammalian orders and has members that are keystone species in most ecosystems of the world. Much of the ecological complexity can be found within the *Muridae* family. This family contains species that serve as reservoirs for all but one (Thottapalyam virus) known hantavirus species and also all but one species of arenavirus. Interestingly, such murid species are subject to large population changes, either as a result of human land-use practices, climate change, or normal population cycles, that pose the most serious threats to human health (Hjelle and Yates, 2001).

The mechanisms for maintenance of hantavirus infections among reservoir rodent populations are not well understood. Infection levels change rapidly as population densities in rodent populations fluctuate. Field studies have demonstrated that hantavirus seroprevalence is highly local, with rodent communities exhibiting 0-1% and as much as 50% prevalence. Seroprevalence at a particular site can vary greatly over time. For most hantaviruses, larger male animals are much more commonly infected than smaller males or females (Hjelle and Yates, 2001).

In a 4-year study of bank voles populations in southern Belgium, PUUV infection in adults was found to be associated with wounds at the end of breeding season, but not in spring. Sexually active animals were significantly more often wounded and positive for infection. Hantavirus infection was associated with higher mobility in juvenile and subadult males. Together with these behavioural and physiologic factors, the habitat also constitutes a crucial element influencing the hantaviral enzootic cycle by determining the distribution of the rodents (Escutenaire *et al*., 2002).

Olsson *et al*. (2002) showed that localised absence of PUUV coincided with the absence of overwintering specimens at several sites during population decline. Long-lived bank voles appear critical to the success of PUUV circulation and persistence within host populations. The chance of being seropositive is population density dependent.

1.6 Evolution of hantaviruses

1.6.1 Methods employed in phylogenetic analysis of viral sequences

The aim of phylogenetic analysis is to arrive at the best possible estimate of the true evolutionary history of the organisms, i.e. their phylogeny. Evolution is the accumulation of change in the genetic makeup of populations over time. As the genetic information is encoded as discrete states (occurrence of A, G, C or T/U in DNA/RNA, or amino acids in the encoded proteins) organised along linear molecules, it is ideally suited for computer analysis. The results of phylogenetic analyses are often presented as "trees", a set of lines (branches) connecting the sequences (tips) via branching points (nodes) (Hungnes *et al*., 2000).

The methods for constructing phylogenetic trees from molecular data can be grouped according to whether the methods uses discrete character states or a distance matrix of pairwise dissimilarities. Distance matrix methods start by calculating a pairwise distance matrix from analysed sequences and then estimate the phylogenetic relationship. In character-state methods, the sequence information is not reduced to the set of pairwise distances but all characters are analysed separately and usually independently from each other (Vandamme, 2003).

In principle, distance methods try to fit a tree to a matrix of pairwise genetic distances. The distance is a single value based on the fraction of positions in which the two sequences differ, defined as p-distance. The p-distance is an underestimation of the true genetic distance because some of the aligned nucleotides are the results of multiple events. Therefore, in distance based methods, one tries to estimate the number of substitutions that have actually occurred by applying a specific evolutionary model that makes assumptions about the nature of evolutionary changes. Correct estimation of genetic distance is crucial and, in most cases, more important than the choice of method to infer the tree topology. Today the most commonly used method to construct distance trees is Neighbor-joining (NJ) method. The NJ method has been proven to be quite efficient in finding the "true" topologies or those that are close. It has the advantage of being very fast, which allows the construction of large trees including hundreds of sequences (Van de Peer, 2003).

The most commonly employed discrete-character methods are parsimony and maximum-likelihood methods. The basic idea of parsimony analysis is simple: one seeks the tree that minimises the amount of evolutionary change required to explain the data. Parsimony methods are most effective when rates of evolution are slow, in other words, the expected amount of change is low (Swofford and Sullivan, 2003). Because this is very often not the case for viral sequences, parsimony methods are in virology not as widely used as NJ or very recently maximum likelihood methods.

Maximum likelihood (ML) analysis is a widely used statistical method that is being applied to a broad range of analyses. In phylogenetic analysis it has been most extensively developed for nucleotide sequence data. With this method, the optimal tree is one that gives the highest probability of observing the actual sequences, given a particular model of evolution. The models allow the specification of probabilities for different kinds of mutations, for example transitions and transversions. ML is a very powerful method when the probabilistic model is realistic (Hungnes *et al*., 2000). Moreover, the ML method intrinsically estimates the standard error on the branch length and therefore gives some statistical support for each branch length and for the entire tree (Vandamme, 2003).

Compared to reality, even the most intricate models of evolution are necessarily simplistic. Thus, complex models are expected to perform better than simpler models, provided that the parameters are realistic. However, as parameters inferred from the small amount of data can be very misleading, in some cases a simple model will perform better than a complex one (Hungnes *et al*., 2000). The determination of an appropriate model can be accomplished using likelihood ratio tests, the Akaike information criterion, Bayesian information criterion or more subjective methods. Particularly the simpler models, such as Jukes and Cantor model (JC69), Kimura 2-parameters model (K80) or even Hasegawa, Kishino, and Yano model (HKY85) should never be used uncritically (Swofford and Sullivan, 2003).

Most of the tree constructing methods are implemented in two most widely used program packages, PHYLIP (Felsenstein, 1993) and PAUP* (Swofford, 2002). In addition, TREE-PUZZLE program for ML analysis (Schmidt *et al*., 2002) is currently gaining the popularity because of its computationally fast quartet puzzling algorithm and additional features as Likelihood mapping analysis and parallel computing.

Recombination is increasingly seen as an important means of shaping genetic diversity in RNA viruses. Given the drastic effect that recombination can have on phylogenetic studies, it is highly desirable that the recombination analysis should be carried out on whole genomes wherever possible, so that putative recombinants can be identified and the possibility of misclassification of strains reduced (Twiddy and Holmes, 2003).

Typical approaches to identify recombination start with splitting the full alignment into a set of smaller overlapping alignments. Phylogenetic analyses are subsequently performed on each of the subalignments in order to see if different regions support different evolutionary histories. Similarity- or dissimilarity-based methods are among the fastest and theoretically least complicated of these methods. The similarity of a sequence to a given set of reference sequences is computed and plotted along the sequence using a sliding window fashion. If recombination results in the crossover between two sufficiently separated evolutionary

lineages, it will be reflected in a plot of the query sequence by a gradual switch of the highest similarity from one reference sequence to another. In a similar sliding-window-based method, bootscaning, a combination of phylogenetic analysis and bootstrap values associated to specific clusters of sequences are used to map recombination breakpoints. Both similarity plots and bootscaning are implemented in the Simplot software (Lole *et al*., 1999), one of the most versatile of the currently available recombination exploration applications (Salminen, 2003).

In addition to these and other graphical applications (e.g. Split Decomposition analysis, TOPAL, PhylPro), more sophisticated procedures exist for locating crossover points (e.g. Informative Sites Analysis, LARD or Homoplasy test) (Worobey and Holmes, 1999).

1.6.2 Phylogenetic analysis of hantaviruses

Hantavirus species are strongly associated with one (or a few closely related) specific rodent species as their natural hosts. Absence of arthropod vector in the virus lifestyle seems to be a crucial in the evolution of hantaviruses and predetermined observed patterns of tight association of these viruses with their specific rodent hosts (Plyusnin and Morzunov, 2001). Phylogenetic analyses of hantaviruses reveal three well-differentiated clades corresponding to viruses circulating in three subfamilies (*Murinae*, *Arvicolinae*, and *Sigmodontinae*) of the rodent family *Muridae* (Figure 3). Moreover, in trees of M and L genes, the viruses of *Arvicolinae* and *Sigmodontinae* form a sister group and the viruses of *Murinae* rodents form outgroup to them. This phylogeny corresponds with a phylogeny of the murid subfamilies based on mitochondrial cytochrome b sequences, supporting the hypothesis that hantaviruses have coevolved with their mammalian hosts at least since the common ancestor of these three subfamilies, which probably occurred about 50 million years ago (MYA) (Hughes and Friedman, 2000).

Similar pattern of branching, reflecting cospeciation, can be observed from the basal nodes to the very fine terminal branches of hantavirus and rodent phylogenetic trees. However, clear pattern of cospeciation could be occasionally disrupted by host-switching events. A typical example could be the association of Khabarovsk virus (KHAV) with *Microtus fortis* and Topografov virus (TOPV) with Siberian lemmings (*Lemmus sibiricus*); while TOPV and KHAV are monophyletic, the respective rodent host species are only distantly related (Plyusnin and Morzunov, 2001; Vapalahti *et al*., 1999).

Evolutionary rate in hantaviruses was estimated using the rate of synonymous substitutions between the S genes from the Old World and New World clusters of viruses which are hosted by members of the genus *Microtus*. It is believed that the first separation of Old and New World *Microtus* occurred in the early Pleistocene, 1.8 – 2.0 MYA (Hoffmann

and Koeppl, 1985). Assuming that the viruses have coevolved with their hosts, the corresponding substitution rate would be 2.41 – 2.68 x 10^{-7} substitutions per site per year. Although considerably lower than synonymous substitution rates of such viruses as influenza or HIV-1, this rate is about two orders of magnitude faster than typical mammalian mutation rate (Hughes and Friedman, 2000).

Although it is currently taken almost as a dogma, Holmes (2003) has recently questioned the virus-host coevolution and cospeciation concept for RNA viruses including hantaviruses. By using the best estimates for rates of evolutionary change (nucleotide substitution) and assuming an approximate molecular clock, it can be inferred that the families of RNA viruses circulating today could only have appeared very recently, probably not more than about 50,000 years ago. Besides the explanations that the molecular clock is not constant in RNA viruses and that the methods currently used to estimate evolutionary distances are flawed in some way, leading to a substantial underestimation of divergence times, the third explanation is that RNA viruses really have a recent origin. The match between virus and host phylogenies that has been taken as evidence for cospeciation over millions of years has to be explained by other mechanisms. One of them might be that the ability to jump species boundaries may be inversely dependent on the phylogenetic distance between hosts, so that it is easier to establish a new infection in a closely related host species than in a more distantly related one.

To determine the extent of homologous recombination in negative-sense RNA viruses, phylogenetic analyses of 35 negative-sense RNA viruses (a total of 2154 sequences) were carried out (Chare *et al*., 2003). Powerful evidence for recombination was found in only five sequences including hantavirus HTNV. In addition, more tentative evidence was found also in PUUV. Evidence for recombination has been documented also for TULV (Sibold *et al*., 1999a; Plyusnin et *et al*., 2002) and some indication was found also for DOBV (chapter 3.2.4; Klempa *et al*., 2003b). However, overall the rates of homologous recombination in including hantaviruses are very much lower than those of mutation. Consequently, recombination does not seem to be a main driving force in the evolution of hantaviruses or other negative-sense RNA viruses (Chare *et al*., 2003).

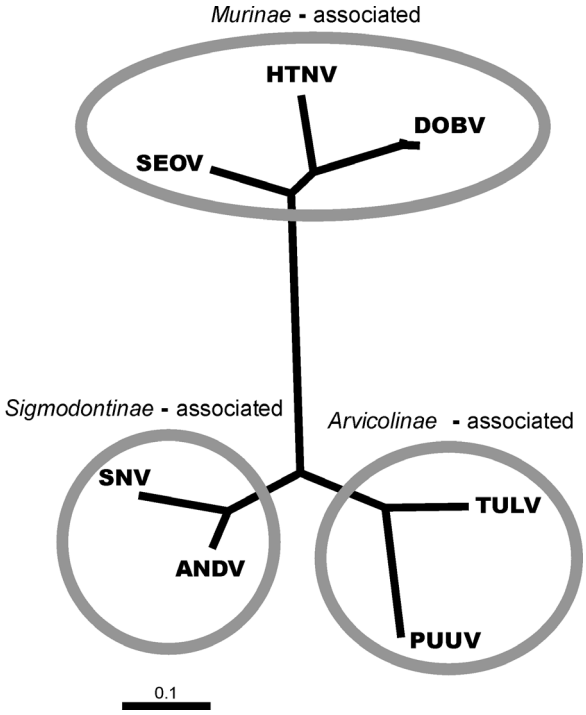

Figure 3: Phylogenetic relationship between the main hantavirus representatives corresponding to the three subfamilies of Murinae rodents.

Maximum likelihood phylogenetic tree (JTT evolutionary model) based on complete N protein amino acid sequence, calculated with TREE-PUZZLE, is shown.

1.7 Dobrava hantavirus

DOBV is intensively studied because of many of its unique properties. DOBV seems to be the most pathogenic European hantavirus. The severity of DOBV- associated HFRS can reach the death rate of up to 12%, as reported in Balkans (Antoniadis *et al*., 1996; Avsic-Zupanc *et al*., 1999; Lundkvist *et al*., 1997c; Papa *et al*., 1998). Most interestingly DOBV is hosted in Europe by at least two different rodent species, *A. flavicollis* (yellow necked mouse) and *A. agrarius* (striped field mouse) (Figure 4). Moreover, there is a hypothesis that the DOBV strains originating from different rodent hosts exhibit different pathogenicity towards humans. These interesting facts led recently to intensive discussions whether the strains originating from *A. agrarius* do represent a unique hantavirus species or only a lineage of original DOBV (Plyusnin and Morzunov, 2001; Nemirov *et al*., 2002; Brus Sjolander *et al*., 2002; Plyusnin, 2002; Plyusnin *et al*., 2003; Klempa *et al*., 2003b, c).

DOBV prototype strain (called here Dobrava/Slovenia, Slo/Af for short) was isolated from lungs of *A. flavicollis* captured in a natural focus of HFRS in Dobrava village, Slovenia. Immunofluorescent antibody assays using convalescent human sera and monoclonal antibodies (MAbs) indicated that DOBV differed from all other recognised hantaviruses but was most closely related to HTNV. Moreover, the part of viral RNA was amplified by RT PCR and in the restriction fragment length polymorphism analysis of the amplified product it was shown to be unique (Avsic-Zupanc *et al*., 1992). Later, the phylogenetic analysis of M and S segments confirmed that DOBV is similar to, but clearly distinct from HTNV and SEOV. Cloning and sequence analysis revealed the M segment to consist of 3644 nt, with a coding capacity of 1134 aa in the virus complementary sense RNA. Seven potential asparagine-linked glycosylation sites were identified in the M segment gene product, one in the G2 and six in the G1 coding regions. The S segment is 1667 nucleotides long, and has a single ORF encoding a protein of 429 aa (Xiao *et al*., 1993; Avsic-Zupanc *et al*., 1995).

Soon, reports about detection of DOBV in other European countries started to appear. DOBV nucleic acid was detected by PCR and sequencing in Greek and Albanian HFRS patients (Antoniadis *et al*., 1996). By Focus reduction neutralisation test (FRNT), DOBV neutralising antibodies were found in patient sera from Bosnia-Herzegovina (Lundkvist *et al*., 1997c), in sera of retrospectively studied HFRS outbreak in Russia (1991-92 in Tula-Ryazan region, Lundkvist *et al*., 1997a), in HFRS patient serum from Germany (Meisel *et al*., 1998), in human sera in Estonia (Lundkvist *et al*., 1998) and Slovakia (Sibold *et al*., 1999b).

Figure 4: *Apodemus flavicollis* (yellow necked mouse) -above- and *A. agrarius* (striped field mouse) -below-, the natural hosts of DOBV (own pictures).

Nineteen HFRS patients due to infection with DOBV from eastern and northern Germany and from Slovakia were investigated. They had shown typical signs of HFRS as fever and other influenza-like symptoms, acute renal failure, trombocytopenia and raise of serum creatinine. DOBV infection was verified by the FRNT where in all cases the sera did neutralise DOBV virus to a significantly higher extent than other virus species including the related HTNV and SEOV. The average age of the patients at the time of disease was 33.8 years; the youngest patient was 15 years old at onset of disease. Interestingly, only 10.5%

(n=2) were females but 89.5% (n=17) were males. Two patients (10.5%) were reported to have developed pulmonary symptoms. No visible haemorrhages occurred. There were no deaths and the clinical course of the HFRS was mild or moderate. Only 18.8% of the patients underwent hemodialysis because of renal failure (Sibold *et al*., 2001).

Surprisingly, DOBV was detected in *A. agrarius* trapped in Estonian islands Saaremaa and Vormsi. Analysis of the partial S segment sequences revealed that they belong to the DOBV genotype and share an ancient ancestor with the Slovenian prototype strain but represent a distinct sublineage. These findings raised the questions whether DOBV can be maintained in both *Apodemus* species and whether the virus exists throughout Europe (Plyusnin *et al*., 1997b).

Subsequently, the virus detected in *A. agrarius* trapped on Saaremaa island in Estonia, was isolated on Vero E6 cells (strain Saaremaa/160V, Saa/160V for short) and its genetic and antigenic characteristics were analysed (Nemirov *et al*., 1999). A cross-neutralisation comparison with the Slovenian prototype strain isolated from *A. flavicollis* revealed 2- to 4-fold differences in the end-point titers of some rabbit and human antisera. When studied with a panel of 25 MAbs, the Estonian (Saa/160V) and Slovenian (Slo/Af) isolates showed similar antigenic patterns that could be distinguished by two MAbs, PUUV N-specific 4E5 (Lundkvist *et al*., 1991) and the anti-HTNV-G1 MAb 8B6 (Arikawa *et al*., 1989). The complete sequences of the S and M segments and partial L segment sequence of the isolate were determined. The phylogenetic analysis confirmed that Estonian strains from *A. agrarius* (Saa/160V and Saa/90Aa) form a well-supported sublineage within the DOBV clade (Nemirov *et al*., 1999). Recently, a new DOBV cell culture isolate was established. DOBV/Ano Poroia (AP/Af) was isolated from an *A. flavicollis* mouse trapped in the North-Eastern Greece. It represents the third DOBV isolate in the world, second obtained from *A. flavicollis* (Papa *et al*., 2001).

DOBV strains in *A. agrarius* were detected also in Slovakia (Sibold *et al*., 1999b), Hungary (Scharninghausen *et al*., 1999) and Russia (Plyusnin *et al*., 1999b), where no casualties have been reported. This led to the idea that *A. flavicollis*-associated strains (Balkans) and *A. agrarius*-associated strains (North-East and Central Europe) exhibit different pathogenicity toward humans (Plyusnin *et al*., 1999b).

The existence of two distinct, rodent host-determined DOBV genetic lineages was definitively confirmed when strains of both lineages were found to occur sympatrically in Slovenia (Avsic-Zupanc *et al*., 2000) and Slovakia (Sibold *et al*., 2001). In Slovenia, partial S and M segment sequences were recovered by RT-PCR from nine *A. flavicollis* and rather exceptionally also from one *A. agrarius*. Sequence comparison and phylogenetic analysis revealed close relatedness and geographical clustering of all *A. flavicollis*-derived virus

sequences. In contrast, the single strain harboured by *A. agrarius* clustered on phylogenetic trees with other DOBV strains derived from *A. agrarius* (Avsic-Zupanc *et al*., 2000). A "complementary" situation was found in the Eastern part of Slovakia, where screening for infected rodents revealed that *A. agrarius* represents the main reservoir for DOBV in this region but a single strain from *A. flavicollis* was also detected. Phylogenetic analysis placed this strain on a *A. flavicollis*-derived lineage (DOBV-Af) whereas other Slovakian strains belong to *A. agrarius*-derived lineage (DOBV-Aa) (Sibold *et al*., 2001).

These interesting findings raised several important questions. On one hand, closely related DOBV lineages are present in two distinct rodent species, on the other hand, two subspecies of *A. agrarius* are harbouring distantly related hantaviruses DOBV (*A. agrarius agrarius*) and HTNV (*A. agrarius matchuricus*). Such a discrepancy might be explained by a host-switch event of (pre)DOBV from *A. flavicollis* to *A. agrarius* (Plyusnin and Morzunov, 2001; Nemirov *et al*., 2002). Moreover, the occurrence of two significantly different lineages steadily linked to the two different host led to the question whether these two DOBV variants represent distinct subtypes or even distinct hantavirus species. This question still remains to be answered and is thoroughly discussed in this work.

In addition, DOBV was recently found in *A. sylvaticus* in South-European Russia and this virus strain could be associated with a severe HFRS case, which occurred in that geographical area (Tkachenko *et al.*, 2001). More sequence data of DOBV strains from *A. sylvaticus* might reveal the definition of third, DOBV-As lineage within DOBV species.

Altogether, DOBV have been detected as an etiologic agent of HFRS in many European countries. Interestingly, most of the reports were only from three regions, Balkans, Central Europe and Russia/Estonia and are summarised in Table 3.

Table 3: The current state of the detection of DOBV. The summary of epidemiological and epizootiological data on DOBV from three regions of Europe. The data which were extended by this work are in bold.

	Balkans	**Russia/Estonia**	**Central Europe**
Neutralising antibodies (FRNT)			
In seroprevalence studies	-	+ [1]	+ [2]
In patients	+ [3]	+ [4]	+ [5]
Clinically characterised patients	Dozens [6]	1 [7]	26 (**3**) [5]
RT-PCR			
Rodents*	Af, Aa [8]	Aa, As [9]	**Aa, Af** [10]
Human	+ [11]	+ [12]	+ [13]
Virus isolation			
Rodents	Slo/Af, AP/Af [14]	Saa/160V [15]	**SK/Aa** [16]
Human	-	-	-

[1] Golovljova *et al*., 2002; Lundkvist *et a*l., 1998
[2] Sibold *et al*., 1999b
[3] Avsic-Zupanc *et al*., 1999; Lundkvist *et al*., 1997c
[4] Lundkvist *et al*., 1997a; Golovljova *et al*., 2000
[5] Meisel *et al*., 1998; Mentel *et al*., 1999; Schütt *et al*., 2001; Sibold *et al*., 2001; Klempa *et al*., 2004; chapters 3.5, 3.6
[6] Lundkvist *et al*., 1997c; Avsic-Zupanc *et al*., 1999; Markotic *et al*., 2002
[7] Golovljova *et al*., 2000
[8] Avsic-Zupanc *et al*., 1992, 2000; Papa *et al*., 2000, 2001
[9] Plyusnin *et al*., 1997b, 1999; Tkachenko *et al*., 2001
[10] Scharninghausen *et al*., 1999; Sibold *et al*., 2001; Klempa *et al*., 2003b; chapter 3.1
[11] Antoniadis *et al*., 1996; Papa *et al*., 1998; Markotic *et al*., 2002
[12] Tkachenko *et al*., 2001
[13] Klempa *et al*., 2004; chapter 3.5
[14] Avsic-Zupanc *et al*., 1992, Papa *et al*., 2001, respectively
[15] Nemirov *et al*., 1999
[16] Klempa *et al*., submitted; chapter 3.4
* Af stands for *Apodemus flavicollis*, Aa for *A. agrarius* and As for *A. sylvaticus*

1.8 Tula hantavirus

In the nineties, a new, PUUV-related hantavirus has been found in European common voles (*Microtus sp.*) and was called Tula hantavirus, TULV (Plyusnin *et al*., 1994; Sibold *et al*., 1995; Vapalahti *et al*., 1996). In the meantime, TULV has been detected in voles from several European regions (Bowen *et al*., 1997; Heyman *et al*., 2002; Plyusnin *et al*., 1995; Sibold *et al*., 1999a). For a long time, TULV was considered to be non-pathogenic towards humans. Only very recently, few data about TULV infections of humans are coming up. However the pathogenic potential of TULV is still not well determined. Moreover, TULV has been attractive to "hantavirologists" and molecular biologists because of the homologous recombination which was detected in TULV strains from East Slovakia (Sibold *et al*., 1999a).

The detection of TULV was for the first time described by Plyusnin *et al*. (1994). Six specimens containing hantavirus antigen related to PUUV and/or PHV, five from *M. arvalis* (Figure 5) and one *M. rossiaemeridionalis* trapped in Tula region, Russia, were found positive in RT-PCR. The sequence analysis of five cloned and sequenced complete S segments showed that the virus is genetically related to but distinct from known members of the *Hantavirus* genus. Immunochemical data obtained for TULV antigen derived from lung tissues of infected rodents and recombinant GST-TUL-N fusion protein confirmed the sequence data. Analysis of TULV with the panel of MAbs revealed that TULV is more related to PUUV and PHV than to HTNV and SEOV but is antigenically distinct from PUUV.

Independently and simultaneously, the TULV sequences were detected also in *M. arvalis* captured in western Slovakia (near Malacky town). Sequence analysis of a major part of the S segment showed this strain, called Malacky, to represent a new subtype within the newly characterised genotype (Sibold *et al*., 1995).

TULV was then detected also in five common voles from Moravia (Czech Republic). In addition to the full length S segment sequences, the proximal part of the M segment (about 1 kb) of two Moravian as well as two Russian strains was sequenced. Phylogenetic analysis suggested a similar evolutionary history for S and M genes of TULV. Comparison of the deduced N protein sequences showed that genetic drift in TULV can occur not only by accumulation of point mutations but also by the deletion of a nucleotide triplet (Ser_{252}). Analysis of naturally expressed TULV N-antigen derived from lung tissue of infected voles with MAbs indicated antigenic heterogeneity among TULV strains (Plyusnin *et al*., 1995).

Subsequently, TULV was isolated from one out of five RT-PCR positive common voles from Moravia, Czech Republic. A Vero E6 cell culture isolate was established after initial passaging of lung samples in laboratory-colonised *M. arvalis*. TULV was defined as a classical serotype by a cross-FRNT. Moreover, serological evidence for a previous TULV

infection was obtained from the serum of a blood donor from Moravia, Czech Republic, showing at least a 16-fold higher titer to TULV as compared to PUUV and other hantaviruses (Vapalahti *et al*., 1996).

Phylogenetic analysis of TULV genetic variants from Slovakia revealed interesting results. In complete S segment phylogenetic tree, strains from Eastern Slovakia clustered with Russian strains and strains from western Slovakia were situated closer to those from Czech Republic. However, phylogenetic analysis of the S segment 3' NCR placed the Eastern Slovakian strains on branch together with Western Slovakian and Czech strains. A bootscan search revealed at least two recombination points in the S sequences of Eastern Slovakian strains which agreed well with the pattern of amino acid substitutions in the N protein and deletions/insertions in the 3' NCR. Altogether, these data suggested that homologous recombination events could play some role in evolution of hantaviruses (Sibold *et al*., 1999a). These findings were confirmed by transfection-mediated generation of functionally competent TULV with recombinant S RNA segment. Independent attempts yielded S RNA molecules of similar structure, carrying a break point located close to one of the break points suggested for natural recombinants (Plyusnin *et al*., 2002).

Figure 5: *Microtus arvalis* (common vole), the natural host of TULV (© Rollin Verlinde – www.natuurbeleving.be)

1.9 Aims of the study

Hantaviruses in Europe are known for many years but the knowledge about their distribution, molecular evolution as well as pathogenic relevance is restricted only to some geographical regions, mostly Fennoscandia and Balkans. The aims of this study were to extend this knowledge to an important area of Central Europe, where DOBV, PUUV as well as TULV have been recently detected.

Currently, Dobrava virus (DOBV) is intensively studied because of its unique properties; different virus lineages exist in different regions of Europe which are harboured by different host reservoirs and which are probably of different virulence towards humans. Although a large overlap between the geographical distribution of *A. agrarius* and *A. flavicollis* exists in Europe, so far the two host-specific subtypes have been detected sympatrically only in the border region between Central and South-East Europe. This detection of DOBV strains from *A. agrarius* and *A. flavicollis* occurring sympatrically in Eastern Slovakia (Sibold *et al.*, 2001) offered an opportunity to remove the effects of geographic isolation on the supposedly host-specific genetic determinants that distinguish DOBV-Af from DOBV-Aa and to examine whether the two virus types are subject to genetic interactions with one another that could influence their evolutionary trajectories. Therefore, the first aim of this work was to extend the work of Sibold *et al*. (2001); to determine complete S and M segment nucleotide sequences of these strains, to analyse their evolutionary history and to study the role of reassortment and recombination processes in hantavirus evolution. In addition, we were intended to identify the candidate host-specific amino acid adaptations in the N proteins and glycoproteins.

By rodent screening, we wanted to identify additional DOBV strains and to infer their phylogenetic relationship. One of the main aims was to establish new DOBV isolates. Particularly an isolate from *A. agrarius* was needed which would better represent the Central European DOBV-Aa lineage than the only isolate from Estonia. For future comparative investigations on experimental virus evolution, virus life cycle, pathogenicity and virus-host interactions it will be crucial to use a viable DOBV-Aa prototype virus.

Because of recent speculations about different pathogenicity of DOBV-Aa and DOBV-Af lineages, we wanted to describe some interesting clinical cases which could contribute to characterisation of DOBV pathogenic potential. Moreover the aim was to obtain viral sequences directly from patient material. The genetic characterisation of viral gene sequences from HFRS patients, enabling molecular classification of the virus strain and providing direct evidence whether DOBV causes HFRS in Central Europe, was still missing. The obtained nucleotide sequence would add some epidemiological relevance to previous

phylogenetic analyses based only on sequences obtained from rodents.

Moreover, our interests were not restricted to DOBV. We wanted to determine not only what hantaviruses are present in rodents populations of Germany and Slovakia but also which of them cause hemorrhagic fever with renal syndrome.

2. MATERIALS AND METHODS

2.1 Trapping of rodents

Between 1994 and 2000, rodents were trapped alive by using Swedish bridge metal traps (Figure 6) in various areas of Slovakia. The trapping sites were selected on the basis of their proximity to reported human cases of HFRS. A total of 50 or 100 traps were set in the late afternoon, in a standardised, systematic manner on the ground, and collected the next morning. The traps were placed mostly at the edges of fields and forests. After collection and prior to dissection the weight, sex, maturity and the exact trapping sites were recorded for each animal. Blood samples were obtained from the *Sinus orbitalis* of deeply anaesthetised rodents. The animals were then sacrificed and dissected for lung, liver and spleen tissues. Serum was separated by centrifugation at 240 g for 10 minutes, and frozen at -20ºC. Tissue samples were stored at -70°C until processed further.

Figure 6: Trapping of small rodents using Swedish bridge metal traps.

2.2 Screening of rodents specimens

2.2.1 Enzyme-linked immunosorbent assay (ELISA)

The rodent sera were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of hantavirus antibodies. For the detection of hantavirus-specific mouse IgG antibodies, an antiglobulin ELISA was performed. Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl/well of recombinant nucleocapsid antigen. Yeast expressed complete N proteins of HTNV strain Fojnica, PUUV strain Vranica-Hällnäs or DOBV strain Slovenia (A. Razanskiene, unpublished data) were diluted in 0.05 M sodium carbonate buffer, pH 9.0, to final concentration of 0.5 µg/ml. Washing of the plates was undertaken five times between each step. After post-coating with blocking buffer (3% BSA in PBS, 100 µl/well) at room temperature for one hour, rodent serum samples diluted 1:200 in PBS with 1% BSA and 0.05% Tween 20 were incubated for 1 hr at 37°C followed by incubation for 1 hr at 37°C with peroxidase-labelled anti-mouse antibody (DAKO Diagnostica, Hamburg, Germany) diluted 1:1000 in PBS with 1% BSA and 0.05% Tween 20. Staining was performed with ready to use TMB substrate (Seramun, Dolgenbrodt, Germany). The reaction was stopped by addition of 1 M sulphuric acid and optical densities of the reaction products were measured at 450/620 nm. Cut-off values were calculated as the mean optical density value plus 3 standard deviations for values of negative controls.

2.2.2 Immunoblotting

Lung tissues samples of *M. arvalis* were screened by immunoblotting for TULV nucleocapsid antigen. Rodent lung tissue samples (2 to 3 mm^3) were homogenised by sonification in 500 ml of Laemmli loading buffer; after denaturation, 15 ml of the homogenate was loaded on a sodium dodecyl sulphate (SDS)–12% polyacrylamide gel and separated by electrophoresis. After transfer of the proteins, the membranes were preadsorbed in 4% non-fat dry milk and subsequently incubated with rabbit polyclonal antibodies (raised against TULV/Malacky recombinant N antigen expressed as a His-tagged protein diluted in PBS–0.05% Tween 20). The indicator antibody was a swine anti-rabbit horseradish peroxidase conjugate used at 1:1000 dilution at 37°C for 1 h. Membranes were washed in PBS–0.05% Tween 20, and the bands were stained with *o*-phenylenediamine dihydrochloride.

2.3 RNA extraction

The RNA of hantavirus antibody/antigen-positive rodents was extracted from lung or liver tissues using the TRIZOL Reagent (GibcoBRL, Invitrogen, Karlsruhe, Germany). This procedure is based on the acid guanidine isothiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Small pieces (2-3 mm^3) of tissues were homogenised in 1 ml of TRIZOL using rotor-stator homogeniser. After 10 min of incubation at room temperature, 200 µl of chloroform was added; the mixture was then mixed by pulse-vortexing for 15 sec and incubated at room temperature for 3 min. After centrifugation at 12000 g for 15 min at 4°C, the aqueous phase was transferred to a fresh tube. 500 µl of cold isopropyl alcohol and 20 µl of 4M Lithium Chloride was added and the mixture was incubated at room temperature for 10 min, followed by centrifugation at 12000 g for 10 min at 4°C. The supernatant was removed and the pellet was subsequently washed with 1 ml of 75% and 100% ethanol, using centrifugation at 7500 g for 3 min at 4°C after every step. The pellet was then air-dried and reconstituted in 20 µl of DEPC water.

For special purposes such as amplification of overlapping fragments of the whole M segment, the QIAgen RNeasy Mini kit (Qiagen, Hilden, Germany) was used. The small pieces of tissues were homogenised in 600 µl of RLT Buffer and the standard RNeasy Mini Protocol for isolation of total RNA from animal tissues was followed according to manufacturer's instructions. RNA was eluted to 30 µl of RNase-free water.

For isolation of hantaviral RNA from cell-culture supernatant, the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used. 280 µl of supernatant was added to 1120 µl of Buffer AVL/Carrier RNA and standard QIAamp viral RNA mini spin protocol was performed. RNA was eluted to 60 µl of Buffer AVE.

2.4 DNA extraction

Total genomic DNA was extracted from small (4 mm^3) pieces of liver or lung tissue using DNeasy Tissue Kit (Qiagen). The tissues were digested for 3 hours or overnight at 55°C in a total volume of 200 µl, including 20 µl proteinase K and 180 µl ATL Buffer, and were subjected to standard DNeasy protocol for animal tissues according to manufacturer's instructions. DNA was eluted to 200 µl of AW2 Buffer from the kit. 5 µl of sample were loaded on gel together with High DNA Mass Ladder to determine approximate yield.

2.5 PCR

2.5.1 Hantavirus initial screening RT-PCR

Total RNA was reverse transcribed with a single genus-specific primer complementary to the 3' and 5' terminal sequences of all three RNA segments (RTS: 5' TAGTAGTAGACT 3') using 200 units of Rnase H- reverse transcriptase Superscript II in RT-buffer (50 mM Tris/HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT; GIBCO, BRL, Gaithersburg, USA) with 500 µM of each dNTP according to manufacturer's instructions. One fourth (5 µl) was then used for the 1st PCR reaction.

A standard nested set of generic primers specific for the hantavirus S RNA genome segment was used for the initial screening. Three different nested PCR systems were used for the detection of PUUV, TULV and DOBV. For primer sequences, see the Table 4. The thermal cycling conditions of both 1st PCRs (40 cycles) and nested PCRs (25 cycles) were: 94°C for 60 sec, 52°C for 60 sec and 72°C for 60 sec, followed by one cycle of final extension for 6 min at 72°C. PCR mixture (50 µl) contained 1.5 mM $MgCl_2$, 200µM of each dNTP , 15 pmoles of each primer pair and 1.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA).

2.5.2 RT-PCR for the sequencing of DOBV complete S and M segments and partial L segment sequence

Amplification of the whole S segment was undertaken by use of the Robust RT PCR System (Finnzymes, Espoo, Finland), following the application protocol of the manufacturers. The single genus-specific primer RT-DOB (5´-ttctgcag TAG TAG TAK RCT CCC TAA ARA G) was used. After 2 min of incubation of 20 pmoles of the primer with 5 µl of RNA at 68°C, the Robust RT PCR mixture was added and after 1 h of incubation at 50°C, 35 cycles of 94°C for 30 sec, 58°C for 45 sec and 72°C for 2 min were performed, followed by one cycle of final extension for 7 min at 72°C.

For sequencing of the complete M segment, the nested RT PCR amplifying overlapping M segment fragments was carried out with the Robust RT PCR System (Finnzymes, Espoo, Finland). Six sets of nested primer sequences were designed from published DOBV entire M segment sequences to generate overlapping cDNA fragments for the entire M genome RNA segment (Table 4). Cycling conditions were similar to those for amplification of the whole S segment, only annealing temperature (50-58°C) and elongation time (1-2 min) were always modified according to melting temperature of primers and length of the amplified fragment.

To obtain the partial L segment sequence, single primer pair DOBL89F (5'-TCA YTG ACA GCA GTR GAR TG) and DOBL669R (5'-AAC ATK GCY TCY ARA GCA GC) (Table 4) amplifying 580 nt long L segment fragment (541 nt when excluding the primer sequences) was designed from published DOBV entire L segment sequences.

2.5.3 PCR of rodent genetic markers

For amplifying rodent genetic markers 12S rRNA and D-loop, PCR was performed. For primer sequences, see Table 4. PCR mixture (50 µl) contained 1.5 mM MgCl2, 200µM of each dNTP , 50 pmoles of each of primer pair and 1.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA). For 12S rRNA, the same cycling conditions as in hantavirus initial screening RT-PCR were used. For D-loop, the annealing temperature of 66°C and 90 sec of elongation were used. 35 cycles were performed.

PCR products were analysed and visualised by electrophoresis in 1.0% agarose gel after 20 min of staining in ethidium bromide (1 µg/ml).

2.6 Cloning and sequencing

The amplified products were cloned into pCR 2.1 - TOPO vector (TA-Cloning kit, Invitrogen, Leek, Netherlands). At least three recombinant plasmids were sequenced in both directions and the consensus sequence from obtained sequences was determined. Dideoxy sequencing was performed on a LICOR sequencer using the Autoread Kit (Pharmacia-Biotech, Freiburg, Germany) as described by the manufacturer.

Table 4: List of PCR primers used for amplification of hantaviral RNA and rodent DNA

PCR	Primer name	Primer sequence
Hantavirus screening PCR		
DOBV-1.PCR	D113 (113-137)	5'-GATGCAGAIAAICAITATGARAA-3'
	D1162c (1142-1162)	5'-AGTTGIATICCCATIGAITGT-3'
nested PCR	D357 357-376)	5'-GAIATTGATGAACCIACAGG-3'
	D955c (935-955)	5'-ACCCAIATTGATGAIGGTGA-3'
PUUV-1.PCR	S1_376 (376-399)	5'-GG(AC)CAGACAGCAGA(CT)TGG-3'
	S2_1252 (1234-1252)	5'-AGCTCAGGRTCCATRTCATC-3'
nested PCR	PS1N (754-776)	5'-ATGGAAAARGARTGCCCMTT-3'
	PS2N (1213-1232)	5'-ACCATYTCYTTKCCCCATTC-3'
TULV-1.PCR	SnMa1 (43-63)	5'-gaggtaccATGAGCCAACTCAAAGAAATA-3'
	SnMa2 (1312-1332)	5'-gaggtaccTTAGATTTTTARYGGTTCCTG-3'
nested PCR	MaS4F (417-440)	5'-CATCACAGGSYTTGCACTTGCAAT-3'
	MaS5C (896-915)	5'-TCCTGAGGCTGCAAGGTCAA-3'
DOBV complete S RT-PCR	RT-DOB (1-22)	5'-ttctgcag TAGTAGTAKRCTCCCTAAARAG-3'
TULV complete S RT-PCR	S10PC (1-29)	5'-gaggtaccgag TAGTAGTAGACTCCCTAAAGAGCTACTAG-3'
DOBV complete M RT-PCR		
1. PCR:	MD TERMINI (1-19)	5'-TAGTAGTAGRCTCCGCAAG-3'
	DM 970R (950-970)	5'-GTTTGCTGCATTTGCAGTGTG-3'
seminested PCR:	MD TERMINI	
	M953R (933-953)	5'-TGTGTGGAATTTTGCCTTCAA-3'
1. PCR:	M905F (905-924)	5'-GTTGCAACTTATTCAATTGC-3'
	M1990R (1970-1990)	5'-TCIGMTGCISTIGCIGCCCA-3'
nested PCR:	G1inC	5´-ACTGCATTCTCAGGAATTCCTAGTTACTCT-3´
	M1490R (1470-1490)	5'-GCCCAICCATGIAAITAICCIGG-3'
1. PCR	M1470c (1469-1488)	5'-CCIGGITTICATGGITGGGC-3'
	M2029R (2009-2029)	5'-CCATGIGCITTITCIKTCCA-3'
nested PCR:	M1674c (1674-1698)	5'-TGTGAIRTITGIAAITAIGAGTGTGA-3'
	M1990R	
1. PCR	M1470c	
	MD3451 (3431-3451)	5'-TACTTATGAGCGCTTATGCTT-3'
nested PCR:	M1674c/MD3451	
1. PCR	MD2240 (2240-2262)	5'-CATTGTTATGGTGCATGTACTAA-3'
	MD TERMINI	
DOBV partial L RT PCR		
1. PCR	DOBL89F (89-108)	5'-TCAYTGACAGCAGTRGARTG-3'
	DOBL669R (650-669)	5'-AACATKGCYTCYARAGCAGC-3'
Rodent genetics		
D-loop	CB1n (15,159-15,184)	5'-GGAGGMCARCCAGTWGAAYACCCATT-3'
	12S1n (72-96	5'-TAATTATAAGGCCAGGACCAAACCT-3'
12S rRNA	L 1091 (490-509)	5'-GGGATTAGATACCCCACTAT-3'
	H 1478 (901-924)	5'-tgactgcaGAGGGTGACGGGCGGTGTGT-3'

The positions of primer binding sites for hantavirus specific PCR refer to the DOBV/Esl862/Aa/97 strain sequences (S segment: Aj269550, M segment: AY168578), for rodent genetics PCR to the *Mus musculus* strain C57BL/6J complete mtDNA sequence. Small letters in primer sequences indicate 5' tails of heterologous sequence integrated for cloning or sequencing purposes.
R=A+G, Y=C+T, M=A+C; S=G+C; K=G+T; W=A+T; I=inozine

2.7 Sequence comparison, phylogeny and recombination analysis

The obtained overlapping nucleic acid sequences were combined for analysis and edited with the aid of the SEQMAN program from the Lasergene software package (DNASTAR, Madison, Wis., USA). The sequence data were further analysed using the BioEdit software package (Hall, 1999). Sequences were aligned using CLUSTALW (Thompson *et al*., 1994) with default parameters. The alignments were then manually checked and corrected where necessary. When aligning the coding sequences, the sequences were first aligned on amino acid level and then reverse-translated to nucleotide sequences using DAMBE software (Xia and Xie, 2001). The reliability of the alignment was checked using DotPlot analysis implemented in BioEdit software package. The alignment was tested for phylogenetic information by Likelihood Mapping analysis (Strimmer and von Haeseler, 1997).

To reconstruct maximum likelihood (ML) phylogenetic trees, we applied quartet puzzling using the TREE-PUZZLE package (Strimmer and von Haeseler, 1997; Schmidt *et al*., 2002). As evolutionary model for the reconstructions we used the Tamura-Nei model, missing parameters were reconstructed from the datasets. The values at the tree branches are the resulting PUZZLE support values, if not otherwise stated. Resulting evolutionary trees were then visualised using TreeView v.1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html)

Bootscan analysis (Salminen *et al*., 1995) was performed with Stuart Ray's SimPlot 2.5 (Lole *et al*., 1999) in combination with the PHYLIP package (Felsenstein, 1993). For the analysis shown we usually used a window size of 200 nucleotides (nt), a step size of 10 nt, and 500 bootstrap samples per window. As underlying phylogeny reconstruction algorithm we used Neighbor-Joining (NEIGHBOR, DNADIST) under the Kimura model with Ts/Tv ratio 2.0 for the distances. For subsequent phylogenetic analysis the alignments were split according to the bootscan diagrams at cross points of a 65% threshold.

For the comparisons, sequence data were obtained from the sequence databases EMBL and Genbank. The list of all hantaviral sequences used in this work together with abbreviated names and accession numbers is shown in Table 5.

Table 5: List of hantavirus strains and their abbreviated names used in sequence analysis

Hantavirus strain	Abbrev. name	Reference	Acc. No.
S segment*			
DOBV/East Slovakia/29Aa/01	Esl/29Aa	Klempa *et al*., 2004; chapter 3.3	AY533118
DOBV/East Slovakia/33Aa/01	Esl/33Aa	Klempa *et al*., 2004; chapter 3.3	AY533119
DOBV/East Slovakia/81Aa/01	Esl/81Aa	Klempa *et al*., 2004; chapter 3.3	AY533120
DOBV/East Slovakia/34Aa/01	Esl/34Aa	chapter 3.4	n.a.
DOBV/East Slovakia/35Aa/01	Esl/35Aa	chapter 3.3	n.a.
DOBV/Slovakia	SK/Aa	chapter 3.4	n.a.
DOBV/East Slovakia/856Aa/97	Esl/856Aa	Sibold *et al*., 2001	AJ269549
DOBV/East Slovakia/862Aa/97	Esl/862Aa	Sibold *et al*., 2001	AJ269550
DOBV/East Slovakia/400Af/98	Esl/400Af	Klempa *et al*., 2003b; chapter 3.2	AY168576
DOBV/East Slovakia/193Aa/01	Esl/193Af	chapter 3.3	n.a.
DOBV/East Slovakia/194Aa/01	Esl/194Af	chapter 3.3	n.a.
DOBV/East Slovakia/197Aa/01	Esl/197Af	Klempa *et al*., 2004; chapter 3.3	AY533121
DOBV/East Slovakia/200Aa/01	Esl/200Af	chapter 3.3	n.a.
DOBV/East Slovakia/201Aa/01	Esl/201Af	chapter 3.3	n.a.
DOBV/H169/02	H169	Klempa *et al*., 2004; chapter 3.5	AY533117
DOBV/Premurkje/Aa-9/96	Slo/9Aa	Avsic-Zupanc *et al*., 2001)	AJ251999
DOBV/Kurkino/44Aa/98	Kurk/44Aa	Plyusnin *et al*., 1999b	AJ131672
DOBV/Kurkino/53Aa/98	Kurk/53Aa	Plyusnin *et al*., 1999b	AJ131673
DOBV/Saaremaa/160v	Saa/160V	Nemirov *et al*., 1999b	AJ009773
DOBV/Saaremaa/90Aa/97	Saa/90Aa	Nemirov *et al*., 1999b	AJ009775
DOBV/Slovenia	Slo/Af	Avsic-Zupanc *et al*., 1995	L41916
DOBV/Ano-Poroia/Af19/1999	AP/Af	Nemirov *et al*., 2003b	AJ410619
DOBV/Dobrava/Af-1/90	DOB-Af1	Avsic-Zupanc *et al*., 2000	AJ251996
DOBV/DOB-PR	DOB-PR	Papa *et al*., 1998	AF060018
DOBV/DOB-EA	DOB-EA	Papa *et al*., 1998	AF060020
DOBV/DOB-SZ	DOB-SZ	Papa *et al*., 1998	AF060022
DOBV/DOB-TD	DOB-TD	Papa *et al*., 1998	AF060023
HTNV/76-118	76-118	Schmaljohn *et al*., 1986	M14626
HTNV/84FLi	84FLi	Liang *et al*., 1994	AY017064
HTNV/LR1	LR1	Yao *et al*., direct submission (d.s.)	AF288294
HTNV/AH09	AH09	Zhihui *et al*., d.s.	AF285264
SEOV/SR11	SR11	Arikawa *et al*., 1990	M34881
SEOV/L99	L99	Liu *et al*., d.s.	AF288299
SEOV/Z37	Z37	Zhihui *et al*., d.s.	AF187082
SEOV/Gou3	Gou3	Wang *et al*., 2000	AB027522
PUUV/Vranica-Hällnäs	PUUV	Reip *et al*., 1995	U14137
TULV/Tula/76Ma/87	Tul/76Ma	Plyusnin *et al*., 1994	Z30941
TULV/Tula/53Ma/87	Tul/53Ma	Plyusnin *et al*., 1994	Z30942
TULV/Tula/175Ma/87	Tul/175Ma	Plyusnin *et al*., 1994	Z30943
TULV/Tula/249Mr/87	Tul/249Mr	Plyusnin *et al*., 1994	Z30944
TULV/Tula/23Ma/87	Tul/23Ma	Plyusnin *et al*., 1994	Z30945
TULV/Malacky/Ma370/94	Mal/Ma370	Sibold *et al*., 1995	Z48235
TULV/Malacky/Ma32/94	Mal/Ma32	Sibold *et al*., 1995	Z68191
TULV/Moravia/5286Ma/94	Mor/5286Ma	Plyusnin *et al*., 1995	Z48573
TULV/Moravia/5293Ma/94	Mor/5293Ma	Plyusnin *et al*., 1995	Z48574

Table 5: List of hantavirus strains and their abbreviated names used in sequence analysis (continued)

Hantavirus strain*	Abbrev. name	Reference	Acc. No.
TULV/Moravia/5294Ma/94	Mor/5294Ma	Plyusnin *et al*., 1995	Z48741
TULV/Moravia/5302Ma/94	Mor/5302Ma	Plyusnin *et al*., 1995	Z49915
TULV/Moravia/5302v/94	Mor/5302v	Vapalahti *et al*., 1996	Z69991
TULV/Koziky/5247Ma/94	Koz/5247Ma	Sibold *et al*., 1999a	AJ223601
TULV/Koziky/5276Ma/94	Koz/5276Ma	Sibold *et al*., 1999a	AJ223600
TULV/109-s	109-s	Scharninghausen,J.J. *et al*., d.s.	AF164094
TULV/g-20S	g-20S	Scharninghausen,J.J. *et al*., d.s.	AF164093
TULV/Kosice/144Ma/95	Kos/144Ma	Sibold *et al*., 1999a	Y13979
TULV/Kosice/667Ma/95	Kos/667/Ma	Sibold *et al*., 1999a	Y13980
TULV/D5	D5	Klempa *et al*., 2003a; chapter 3.7	AF289819
TULV/D17	D17	Klempa *et al*., 2003a; chapter 3.7	AF289820
TULV/D63	D63	Klempa *et al*., 2003a; chapter 3.7	AF289821
TULV/Lodz-2	Lodz-2	Song,J.-W. *et al*., d.s.	Af063892
TULV/Lodz-1	Lodz-1	Song,J.-W. *et al*., d.s.	Af063897
ISLAV/PC-SB-77	PC-SB-77	Song *et al*., 1995	U31535
ISLAV/MC-SB-1	MC-SB-1	Song *et al*., 1995	U31534
ISLAV/MC-SB-47	MC-SB-47	Song *et al*., 1995	U19302
PHV/prototype	PHV	Parrington and Kang, 1990	M34011
PHV/PH-1	PH-1	Plyusnin *et al*., 1996	Z49098
PHV/PH-NY1	PH-NY1	Huang,C. *et al*., d.s.	U47136
TOPV/Ls136V	TOPV	Vapalahti *et al*., 1999	AJ011646
KHAV/MF-43	KHAV	Horling *et al*., 1996	U35255
BAYV/prototype	BAYV	Morzunov *et al*., 1995	L36929
NYV/RI-1	NYV	Hjelle *et al*., 1995	U09488
SNV/NM H10	SNV	Spiropoulou *et al*., 1994	L25784
ELMCV/RM-97	ELMCV	Hjelle *et al*., 1994	U11427
M segment			
DOBV/East Slovakia/862Aa/97	Esl/862Aa	Klempa *et al*., 2003b; chapter 3.2	AY168578
DOBV/East Slovakia/400Af/98	Esl/400Af	Klempa *et al*., 2003b; chapter 3.2	AY168577
DOBV/Slovakia	SK/Aa	chapter 3.4	n.a.
DOBV/Saaremaa/160V	Saa/160V	Nemirov *et al*., 1999	AJ009774
DOBV/Slovenia	Slo/Af	Avsic-Zupanc *et al*., 1995	L33685
DOBV/Ano-Poroia/Af19/1999	AP/Af	Nemirov *et al*., 2003b	AJ410616
HTNV/76-118	76-118	Schmaljohn *et al*., 1986	M14627
HTNV/84FLi	84FLi	Liang *et al*., 1994	AF366569
HTNV/LR1	LR1	Yao *et al*., d.s.	AF288293
HTNV/AH09	AH09	Zhihui *et al*., d.s.	AF285265
SEOV/SR11	SR11	Arikawa *et al*., 1990	M34882
SEOV/L99	L99	Liu *et al*., d.s.	AF035833
SEOV/Z37	Z37	Zhihui *et al*., d.s.	AF187081
SEOV/Gou3	Gou3	Wang *et al*., 2000	AB027521
PUUV/Vranica-Hällnäs	PUUV	Reip *et al*., 1995	U14136
SNV/NM H10	SNV	Spiropoulou *et al*., 1994	L25783
ELMCV/RM-97	ELMCV	Hjelle *et al*., 1994	U26828

Table 5: List of hantavirus strains and their abbreviated names used in sequence analysis (continued)

Hantavirus strain*	Abbrev. name	Reference	Acc. No.
TULV/Moravia/5302v/95	TULV	Vapalahti *et al*., 1996	Z69993
L segment			
DOBV/Saaremaa/160V	Saa/160V	Nemirov *et al*., 2003b	AJ410618
DOBV/Ano-Poroia/Af19/1999	AP/Af	Nemirov *et al*., 2003b	AJ410617
DOBV/Slovenia	Slo/Af	Nemirov *et al*., 1999	AJ009779
DOBV/Slovakia	SK/Aa	chapter 3.4	n.a.
HTNV/76-118	76-118	Schmaljohn *et al*., 1990	NC_005222
SEOV/80-39	80-39	Antic *et al*., 1991	NC_005238
PUUV/ CG1820	PUUV	Stohwasser *et al*., 1991	M63194
TULV/Moravia/5302v/95	TULV	Vapalahti *et al*., 1996	AJ005637
SNV/NM H10	SNV	Spiropoulou *et al*., 1994	L37901
ANDV/Chile-9717869	ELMCV	Meissner *et al*., 2002	AF291704

* For hantavirus species abbreviations see Table 2.
n.a., not available
d.s., direct submission

2.8 Virus isolation

DOBV RT-PCR-positive lung and liver samples from two naturally infected, seropositive *A. agrarius* trapped in Rozhanovce, Eastern Slovakia, were used for virus isolation attempts. The samples were processed as 10% tissue suspensions in Dulbecco's medium supplemented with 0.2% bovine serum albumin (BSA). The tissues were triturated in a closed mechanical blender FastPrep Instrument (BIO 101 Systems, Carlsbad, CA, USA). Triturated tissues were briefly centrifuged at low speed to remove larger tissue fragments and inoculated (0.4 ml/flask) onto cultures of confluent Vero E6 cells in 25 cm^2 flasks (three for each sample). Virus was then allowed to adsorb at 37°C. The cell culture medium (MEM plus 10% fetal calf serum, L-glutamat, penicillin and streptomycin) was changed for the first time after 90 min and then weekly. At 2-3 week intervals, cells, detached by trypsin treatment, were passaged into new culture flask with the addition of the same amount of fresh uninfected cells according to a recently described protocol (Nemirov *et al*., 1999). While suspended, several slides were prepared and examined for characteristic hantavirus antigen expression following immunofluorescence assay (IFA) techniques. The experiments were performed under biosafety level 3 containment conditions in the Institute of Virology, Charité School of Medicine.

2.9 Immunofluorescence assay

2.9.1 Preparation of slides for indirect immunofluorescence assay (IFA)

Cells were prepared on Teflon-coated slides with 12 circular areas ("spots"). After detaching with trypsin treatment, the cells were suspended in cell culture medium and washed twice. Washed cells from every flask were resuspended in 5 ml of cell culture medium and 20 µl of the suspension was deposited on each spot of the slides cleaned with 70% ethanol. The slides were put in a moist chamber and incubated in at 37°C and 5% CO_2 overnight. The slides were washed two times in PBS and once in bidistillated water and anhydrous acetone and then fixed in anhydrous acetone at 4°C for 10 minutes, air-dried and used or stored at -20°C till next day.

2.9.2 Staining of slides

Slides were first washed in PBS and air-dried. 20 µl of DOBV convalescent antisera pool diluted 1:200 in PBS were deposited on every spot and incubated at room temperature

for 30 min in a moist chamber. The slides were washed, with 3 changes of PBS each for 5 min, and air-dried. Fluorescein isothiocyanate (FITC)- conjugated anti-human immunoglobulin, diluted 1:40 in PBS, was added, 20 µl to each spot, and the slides were returned to the moist chamber at room temperature for 30 min. To increase the easy of viewing, Evan's blue at dilution 1:1500 was added to the conjugate. The slides were again washed, with three changes of PBS each for 5 min, and air-dried. The slides were mounted with the mounting medium (Progen) under cover slips and examined for characteristic cytoplasmic pattern in a fluorescence microscope.

For the antigenic characterisation of the new isolate, a panel of eight monoclonal antibodies (mAbs) directed against N protein of different hantaviruses was used. The reactivity of the mAbs E5/G6, Eco2, C16D11, C24B4, B5D9 (Yoshimatsu *et al*., 1996; Zöller *et al*., 1993) and 2E2 (gift of J. Groen) directed against HTNV, R31 against SEOV (Progen Biotechnik, GmbH Heidelberg, Germany) and 5C2/E10 against ANDV (Immunological and Biochemical Testsystems GmbH, Reutlingen, Germany) was analysed in parallel using IFA slides with Vero E6 cells infected with DOBV strains SK/Aa and Slo/Af. After 10 days of incubation the infected cells were mixed with the uninfected cells at a ratio of 1:2. The slides were then stained as described above but FITC-conjugated anti-mouse immunoglobulin was used instead of anti-human conjugate. A positive reaction was stated to be specific if at least one third of the cells showed the fluorescence signal.

2.10 Virus titration

The viral stock, prepared from cell-culture supernatant of infected Vero E6 cells, was titrated using the chemiluminiscent focus assay as described by Heider *et al*. (2001). From the six-well plates with nearly confluent monolayer of Vero E6 cells, the cell-culture growth medium was discarded and the cells were inoculated with 0.2 ml/well of tenfold dilutions of viral stock in Hanks' balanced salt solution (HBSS; GibcoBRL) supplemented with 2% HEPES (GibcoBRL), 2% fetal calf serum (FCS) and antibiotics mixture PSN (GibcoBRL). After the incubation for 1 h at 37°C in a humidified 5% CO_2 atmosphere, the cells were overlaid with 2.5 ml/well of a pre-warmed (42°C) mixture of one part 1% agarose in water and one part overlay medium (2x Eagle's basal medium with Earle's salt and L-glutamine, GibcoBRL) and the plates were then incubated for 10 days under the conditions described above.

Gently injecting 2–3 ml of methanol under the agarose layer and turning the plate upside down then removed the agarose. The cells were fixed with 2 ml/well of methanol for 8

min, allowed to dry and gently washed three times with washing buffer (PBS supplemented with 0.15% Tween 20, Boehringer Ingelheim, Heidelberg). 1 ml/well of anti-DOBV human convalescent serum diluted 1:1000 in washing buffer was added and the plates were incubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere and then five times washed with washing buffer. 1 ml/well of goat anti-human IgG conjugated with horseradish peroxidase and diluted 1:1000 was added and after incubation for 1 h at 37°C in a humidified 5% CO_2 atmosphere, the cells were five times washed. Immediately after adding 0.5 ml/well of the chemiluminescence substrate (Super Signal West Dura Extended Duration Substrate, Pierce, Rockford, USA) diluted 1:5 in water, the plates were evaluated in DIANA Chemiluminescence System (Raytest, Straubenhardt, Germany). On a light screen, the foci of infected cells were enumerated as black-coloured dots.

2.11 Chemiluminiscent focus reduction neutralisation test (c-FRNT)

c-FRNT was performed basically following the protocol described above for the virus titration (Heider *et al*., 2001). Human convalescent sera were first diluted serially, mixed with an equal volume containing 30–80 focus forming units (FFU) of the respective virus and incubated for 1 h at 37°C prior inoculation the cells. After 10 days of incubation, DOBV-specific rabbit antiserum and goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) was used to detect the viral antigen in infected cells. An at least 80% reduction in the number of foci was considered as the criterion for virus neutralisation.

3. RESULTS

3.1 Screening of rodents from Slovakia

A total of 1733 rodents representing nine species were collected between 1995 and 2001 in Slovakia (Figure 7). Among them, 1032 animals belonged to *Murinae* subfamily and 701 to *Arvicolinae* subfamily of the *Muridae* family. The three most common species, *A. flavicollis, A. agrarius* and *Clethrionomys. glareolus*, comprised 83.5% of captures. However, *A. agrarius* was found exclusively in East Slovakia whereas *A. flavicollis* and *C. glareolus* were distributed over the whole country.

Antibodies reactive with hantavirus antigen (PUUV, HTNV and/or DOBV) were detected in six species of *Muridae* rodents (Table 6). However, in subsequent analyses we were able to detect viral RNA by RT PCR only in four species, *A. agrarius* (DOBV), *A. flavicollis* (DOBV), *C. glareolus* (PUUV) and *M. arvalis* (TULV). Prevalence of antibodies reactive to hantavirus antigen was highest in *A. agrarius* (13.7%), followed by *M. arvalis* (11.6%) and *A. flavicollis* (3.9%). However, the seroprevalence was highly variable, especially in case of *M. arvalis*, where 12 out 15 positive mice were captured during years 1995-96. Nevertheless, the data show that DOBV circulating in *Apodemus* mice represents the most common hantavirus in Slovakia.

Of 984 rodents tested in the western Slovakia, 36 (3.7%) were sero-positive. In Eastern Slovakia we found 75 (10.0%) positives among 749 rodents (Table 6). This difference was significant (chi-square = 28.65) but can be explained by geographical distribution of *A. agrarius* which represents the most frequently infected species in Slovakia and can be found only in Eastern part of the country. After excluding the data of *A. agrarius* from the analysis, the distribution was not significant (chi square = 0.04). 39 individual sites were surveyed in Western (11 sites) and Eastern part (28 sites) of Slovakia. Antibody prevalence at individual sites varied from 0.0 to 26.0%. Three sites with highest antibody prevalence, Slanske Nove Mesto (26.0%), Cerhov (24.4%) and Rozhanovce (21,7%), are all in Eastern Slovakia, what can be also explained by presence of *A. agrarius* at those localities. Hantavirus antibody prevalence was 7.58% in males and 5.07% in females. This difference was significant (chi-square = 4.53).

Figure 7: Map of Central Europe.

The green dots indicate the regions of trapping activities in Slovakia (Chapter 3.1). The red dot indicates the place where the first patient-associated DOBV-Aa sequence have been obtained (Chapter 3.5). The blue dot indicates the area where a TULV-associated HFRS case has been reported and TULV sequences from *Microtus arvalis* rodents have been obtained (Chapter 3.7).

Table 6: Prevalence of antibodies to HTNV, PUUV and/or DOBV among wild rodents in Slovakia, 1995-2001.

	Eastern Slovakia			Western Slovakia			Slovakia (total)		
Subfamily/ Species	No. Tested	No. Pos.	% Pos.	No. Tested	No. Pos.	% Pos.	No. Tested	No. Pos.	% Pos.
Murinae									
A. agrarius	467	64	13.7	0	-	-	467	64	13.7
A. flavicollis	119	2	1.7	290	14	4.8	409	16	3.9
A. microps	42	1	2.4	1	0	0	43	1	2.3
A. sylvaticus	1	0	0	103	1	1.0	104	1	1.0
Mus musculus	2	0	0	6	0	0	8	0	0.0
Micromys minutus	1	0	0	0	0	-	1	0	0.0
SUBTOTAL	632	67	10.6	400	15	3.8	1032	82	7.9
Arvicolinae									
Clethrionomys glareolus	58	2	3.4	513	12	2.3	571	14	2.5
Microtus arvalis	59	6	10.2	70	9	12.9	129	15	11.6
Pitymys subterraneus	0	-	-	1	0	0.0	1	0	0.0
SUBTOTAL	117	8	6.8	584	21	3.6	701	29	4.1
TOTAL	**749**	**75**	**10.0**	**984**	**36**	**3.7**	**1733**	**111**	**6.4**

3.2 Genetic analysis of complete S and M segment sequences from distinct Dobrava hantavirus subtypes of *Apodemus agrarius* and *A. flavicollis*

3.2.1 Comparison of S segment sequences

We have established the total nucleotide sequence of the S segment of the first *A. flavicollis* – derived DOBV strain from Central Europe, DOBV/Esl/400Af/98 (Esl/400Af). In contrast to the length of 1,704 nts as determined for *A. agrarius* - derived DOBV/Esl/856Aa/97 (Esl/856Aa) and DOBV/Esl/862Aa/97 (Esl/862Aa) (Sibold *et al.*, 2001), the S segment of the Esl/400Af strain was found to encompass only 1,673 nt.

Table 7 (upper part) shows the nucleotide and amino acid percent identities between the N-encoding ORF sequences and deduced N proteins of different representatives of the DOBV species, respectively. Within the DOBV strains, Esl/856Aa and Esl/862Aa exhibited

the lowest nucleotide sequence similarities (about 85 - 87 %) to the two DOBV-Af strains (Esl/400Af and DOBV/Slovenia-Af) as well as to the two DOBV/Saaremaa-Aa strains (Saa/160v and Saa/90Aa; Nemirov *et al*., 1999). The East European DOBV/Kurkino-Aa strains (Kur/44Aa and Kur/53Aa; Plyusnin *et al*., 1999b) are somewhat more related to Esl/856Aa and Esl/862Aa (about 90 % nt identity). On the amino acid level, Esl/856Aa and Esl/862Aa showed the lowest similarity to Saaremaa-Aa strains, being even lower than to the DOBV-Af strains. As expected, Esl/400Af exhibited high nt and aa similarities to DOBV/Slovenia-Af (Slo/Af) but lower similarity to the DOBV-Aa strains (Esl/856Aa, Esl/862Aa, Kur/44Aa, Kur/53Aa, Saa/160v, and Saa/90Aa).

The single ORF (nt 36 - 1,325) for the viral N protein encodes a putative protein of 429 aa length. In contrast to PUUV and TULV, no putative ORF2 in the S segment of the DOBV-Aa or DOBV-Af strains analysed was found. In general, the N-terminal half of the N protein (approximately 200 aa) is more conserved than the C-terminal half. Cysteine residues at aa positions 219, 244, 293, 315 and 319 are highly conserved; their functional importance is also suggested by conservation in all hantaviruses analysed here (aa 219, 244 and 315), in all *Muridae*-derived hantaviruses (aa 293), and in all Old World hantaviruses (aa 319).

The 3' non-coding region (3'NCR) of the S segment of strain Esl/400Af is 348 nt long and appears highly similar to Slo/Af but most different from Esl/856Aa and Esl/862Aa (Table 7, lower part). The 3'NCR sequences of Esl/856Aa and Esl/862Aa showed identities of about 84-85% to the other DOBV-like strains (no matter whether of *A. agrarius* or *A. flavicollis* origin). Interestingly, the 3´NCR of Esl/856Aa and Esl/862Aa is longer than that of Esl/400Af; this is due to an 32 nt long insertion identified 28 bp downstream the stop codon in both Esl/856Aa and Esl/862Aa strains. The insert is (imperfectly) repetitive with its downstream neighbouring tract. Besides East Slovakian strains Esl/856Aa and Esl/862Aa, no other available DOBV S segment sequences contain this insertion in their 3´NCR.

Altogether, whereas S segments of the two DOBV-Af strains were found to be very similar, the DOBV-Aa strains exhibited higher diversity. The Central and East European strains were found to be rather dissimilar from the Saaremaa-Aa strains.

Table 7: S segment nucleotide and amino acid percent identity of DOBV strains, HTNV, and SEOV

ORF										
Strain*	Esl/856Aa	Esl/862Aa	Kur/53Aa	Kur/44Aa	Saa/160V	Saa/90Aa	Esl/400Af	Slo/Af	HTNV	SEOV
Esl/856Aa	-	99.6	90.5	90.2	87.0	87.4	85.0	86.7	74.0	73.5
Esl/862Aa	99.5	-	90.4	90.2	86.9	87.3	85.0	86.7	74.0	73.6
Kur/53Aa	98.6	98.6	-	99.7	87.8	87.7	87.6	88.1	74.2	74.2
Kur/44Aa	98.1	98.1	99.5	-	87.5	87.4	87.4	87.8	74.0	74.1
Saa/160V	96.7	96.7	96.2	95.8	-	98.3	87.5	87.9	73.3	72.5
Saa/90Aa	96.5	96.5	96.0	95.5	99.7	-	87.2	88.0	73.1	72.8
Esl/400Af	97.6	97.6	97.2	96.7	97.2	96.9	-	95.3	73.8	72.5
Slo/Af	97.9	97.9	97.2	96.7	97.2	96.9	99.0	-	74.2	73.6
HTNV	82.9	82.9	83.4	83.2	82.5	82.2	82.7	82.7	-	73.9
SEOV	80.8	80.8	81.3	80.8	79.9	79.9	80.1	80.1	82.2	-
3´NCR										
Strain*	Esl/856Aa	Esl/862Aa	Kur/53Aa	Kur/44Aa	Saa/160V	Saa/90Aa	Esl/400Af	Slo/Af	HTNV	SEOV
Esl/856Aa	-	100	85.3	85.3	83.6	84.1	84.4	83.6	51.3	40.7
Esl/862Aa		-	85.3	85.3	83.6	84.1	84.4	83.6	51.3	40.7
Kur/53Aa			-	100	89.0	90.0	91.8	90.3	53.7	40.6
Kur/44Aa				-	89.0	90.0	91.8	90.3	53.7	40.6
Saa/160V					-	98.7	89.6	89.9	53.6	40.6
Saa/90Aa						-	90.2	89.9	53.8	40.6
Esl/400Af							-	96.6	54.4	40.1
Slo/Af								-	54.7	39.7
HTNV									-	46.3
SEOV										-

The S segment ORF (upper part) and 3´NCR (lower part) were analysed. The identity values were calculated using Clustal method. The percentage differences for nucleotide (above the diagonal) and amino acid (below the diagonal) sequences are presented.
*For strain abbreviations, see Table 5.

53

3.2.2 Comparison of M segment sequences

Total M segment nucleotide sequences have been determined for the sympatrically occurring strains Esl/862Aa and Esl/400Af from East Slovakia. Their lengths were found to be 3,643 and 3,648 nt, respectively. Both strains encode a putative glycoprotein precursor (GPC) consisting of 1,135 aa.

Table 8 (upper part) shows the degree of nucleotide and deduced amino acid sequence identity between the glycoprotein-encoding ORFs of these two strains and the two other DOBV strains for which complete M segment sequences are known, DOBV/Saaremaa/160v (Saa/160v) and DOBV/Slovenia-Af (Slo/Af). Strain Esl/400Af resembles the DOBV/Slovenia-Af prototype sequence (93.0% nt and 99.2 % aa identity), whereas greater differences were found between Esl/862Aa and Saa/160v (87.3% nt and 96.1% aa identity) and, even more pronounced, between Esl/862Aa and the DOBV-Af strains (only about 82.5 % nt and 94.1 % aa identity).

Similar relationships could be deduced from the comparative analysis of the 3´NCR of the M segment (Table 8, lower part). However, the M segment 3´NCR sequences exhibit a higher variability than the S segment 3´NCRs of the strains analysed.

In contrast to the S segment analysis, where the Central European DOBV-Aa strains exhibited a similar level of identity with Saaremaa-Aa strains and DOBV-Af strains each, the M segment of our Central European strain Esl/862Aa showed higher similarity to Saa/160v than to the DOBV-Af strains. In addition, the M segments of the DOBV-Af strains appeared more similar to each other than were the DOBV-Aa M segments to one another.

The putative aa sequences of the GPC of Esl/Aa862 and Esl/Af400 showed the typical sequence motifs of hantavirus glycoproteins. At the N-terminus of the GPC a signal peptide of 18 aa could be predicted. The GPC of both strains contain the highly conserved WAASA motif responsible for co-translational cleavage of G1 and G2. The number (n = 60; 5.3% of total aa) and localisation of cysteine residues are identical for all four compared DOBV-like sequences, 43 of them can be found in all available hantavirus GPC sequences. The YRTL motif, a potential internalisation signal for the endoplasmic reticulum (ER), was found in the cytoplasmic domain of G1 in all analysed DOBV sequences. KHKKS, a potential ER retention signal in the cytoplasmic domain of G2, is modified in all DOBV sequences to KHKRS.

Table 8: M segment nucleotide and amino acid percent identity of DOBV strains, HTNV, and SEOV

ORF						
Strain*	Esl/862Aa	Saa/160V	Esl/400Af	Slo/Af	HTNV	SEOV
Esl/862Aa	-	87.3	82.6	82.5	70.5	70.8
Saa/160V	96.1	-	82.2	82.4	71.2	70.6
Esl/400Af	94.1	94.3	-	93.0	71.0	70.6
Slo/Af	94.1	94.5	99.2	-	70.8	70.7
HTN	77.0	76.9	77.7	77.5	-	72.3
SEO	76.8	76.4	77.3	77.5	76.8	-
3´NCR						
Strain*	Esl/862Aa	Saa/160V	Esl/400Af	Slo/Af	HTNV	SEOV
Esl/862Aa	-	79.7	62.5	63.1	37.8	44.2
Saa/160V		-	62.0	63.6	41.3	44.2
Esl/400Af			-	91.9	40.5	43.2
Slo/Af				-	42.3	44.5
HTNV					-	39.1
SEOV						-

The M segment ORF (upper part) and 3´NCR (lower part) were analysed. The identity values were calculated using Clustal method. The percentage differences for nucleotide (above the diagonal) and amino acid (below the diagonal) sequences are presented.
*For strain abbreviations, see Table 5.

3.2.3 Phylogenetic trees and proof of reassortment

The molecular phylogeny of complete S segment nucleotide sequences from the DOBV strains by maximum likelihood (ML) analysis clearly indicated that Central European DOBV-Aa strains form a well-supported monophyletic group with the Russian Kurkino-Aa strains whereas the strain Esl/400Af forms a monophyletic group together with Slo/Af (Figure 8). Using the complete S segment sequence for analysis, the PUZZLE support for position of the Saaremaa-Aa strains within the tree was slightly below the 70% threshold limit. Therefore we repeated the analysis on the basis of ORF nucleotide sequences (Figure 8, upper right) and aa sequences of the N proteins (data not shown) of the same virus strains. These analyses unambiguously grouped with high support Saaremaa-Aa sequences together with the DOBV-Af strains and well-apart from the Central European and Russian DOBV-Aa lineages. This shows that the S segment of Saaremaa-Aa strains is related to the S segment

of *A. flavicollis*-derived DOBV strains. This result is supported also by presence of five characteristic amino acid exchanges in the N protein where both Saaremaa-Aa strains exhibit the same amino acid as Esl/400Af and/or Slo/Af in contrast to Central European and Russian strains (data not shown). In the opposite direction, only at three positions Saaremaa-Aa strains exhibit aa residues different from those of DOBV-Af strains and identical to those of East and Central European DOBV-Aa strains (see following chapter).

Figure 9 shows the phylogenetic tree based on available complete M segment sequences of DOBV strains and other hantaviruses. In addition to our two newly determined complete sequences Esl/862Aa and Esl/400Af only two other complete M segment sequences were available for analysis. However, the results clearly show that the Esl/862Aa strain forms one well-supported group with Saa/160v whereas the strain Esl/400Af demonstrated, as expected, a close relationship to the Slo/Af prototype. This is also supported by presence of 38 aa exchanges in the GPC where Esl/862Aa and Saa/160v carry identical amino acid residues in contrast to both DOBV-Af strains.

The difference in the phylogenetic placement of Saaremaa-Aa in respect to S and M segments shows that this virus was involved in reassortment processes during its evolution; its S segment is more related to DOBV-Af whereas its M segment resembles the Central European strain Esl/862Aa.

To investigate the possibility that this contradiction of S- and M-segment derived trees could have been caused simply by absence of additional sequences (e.g., Kurkino-Aa strains) in the M but not S segment ML analysis, we repeated the S segment analysis without involving sequences of Kurkino-Aa strains. Nevertheless, also under these conditions the phylogenetic grouping of the S segments of Saaremaa-Aa strains with DOBV-Af remained unchanged (data not shown) confirming the above mentioned results.

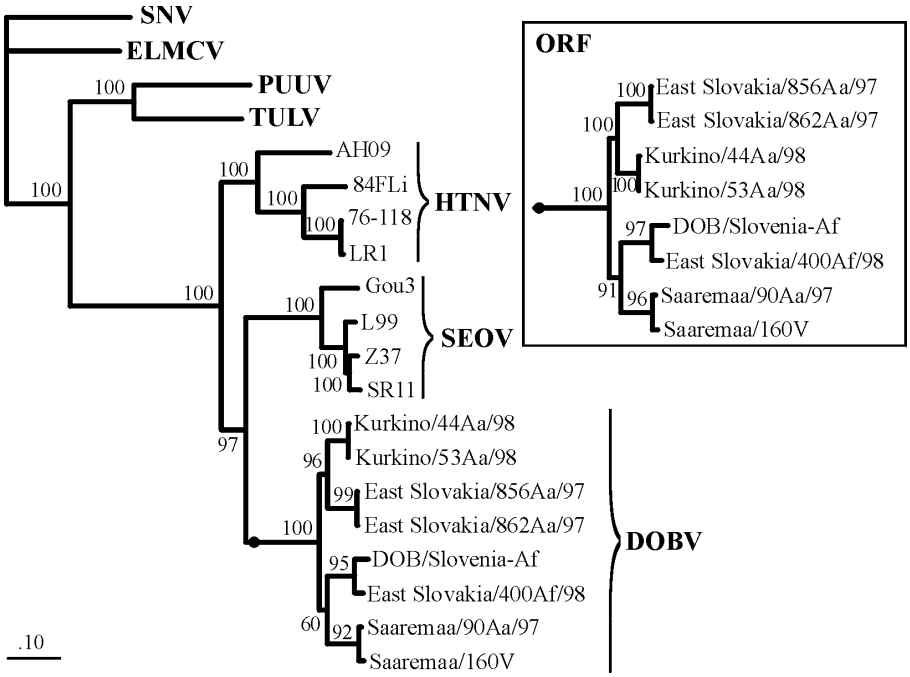

Figure 8: Phylogenetic ML trees from the complete S segment nucleotide sequences of DOBV and further hantaviruses, computed with TREE-PUZZLE.

The main tree is based on sequences of the complete S segments. The insert shows the subtree of the complete ORF sequences for the DOBV-like strains. The sources of hantavirus sequences are given in Table 5.

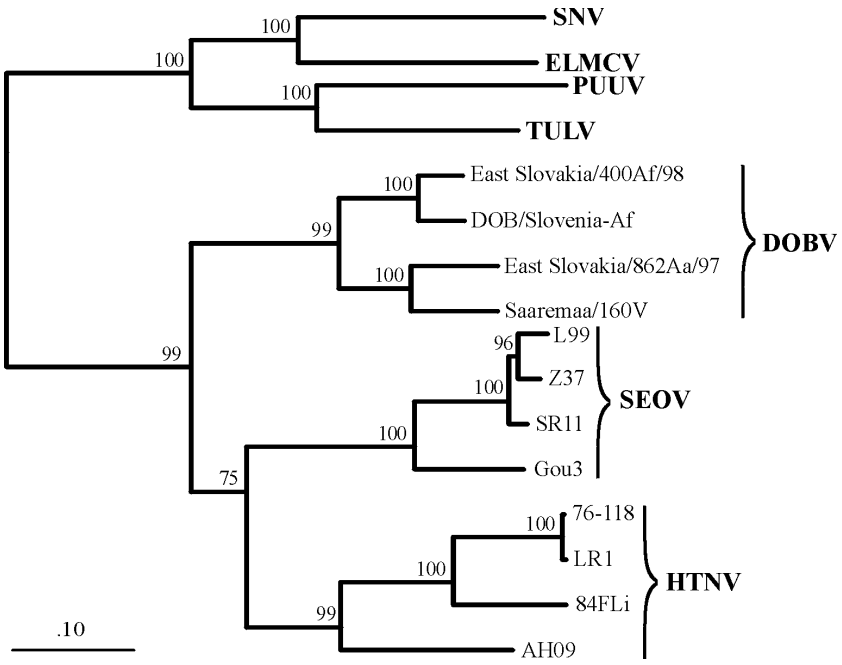

Figure 9: Phylogenetic ML tree of the available complete M segment nucleotide sequences of different members of the DOBV lineage and further hantaviruses computed with TREE-PUZZLE.

The sources of hantavirus sequences are given in Table 5.

3.2.4 Recombination analysis

To study more precisely the evolutionary history of the two genomic segments we performed bootscanning analyses on the alignments of the S and M segment ORFs for the viruses Esl/400Af, Esl/856Aa, and Esl/862Aa. In all initial bootscanning analyses (data not shown) as well as all phylogeny reconstructions, Esl/400Af formed a highly supported subtree together with Slo/Af, as did Esl/856Aa with Esl/862Aa. So we assigned the former as close relatives to one group ”DOBV-Af” and the latter to a group here called ”Esl/8xxAa”.

Subsequent bootscanning analyses of the Esl/8xxAa group (S segment, not shown) or Esl/862Aa (M segment, Figure 10) as query, showed the following principal results. While Esl/862Aa revealed a high relationship to Saa/160v in most parts of the M segment ORF, the relationship of Esl/8xxAa to Kurkino-Aa strains throughout the S segment ORF gained only low support values (Figure 10). To elucidate the specific evolutionary histories of different regions in the S and M segments of the Esl/8xxAa viruses we split the full alignments into subalignments guided by the main cross points of a 65% threshold in the bootscan diagrams. For the M segment analysis these split points are marked above the bootscan diagram in Figure 10. The subalignments were then used to reconstruct ML phylogenies using quartet puzzling. The resulting DOBV subtrees were then further examined. The M segment subtrees are given in the upper part of Figure 10.

The bootscan analysis of Esl/8xxAa in respect to the S segment ORFs gave support for the Esl/8xxAa – Kurkino-Aa monophyly in most parts of the sequence although with very low support values. The values almost never reached 90% and were mostly below 60% (data not shown). Changes of the evolutionary history were suggested by substantial drops of the curve associated with Kurkino-Aa for the regions from nt 400-600 and nt 730-1080. The ML phylogenies reconstructed from the subalignments of the S segment ORFs revealed changes in the evolutionary history along the sequences. While the trees grouped together Esl/8xxAa and Kurkino-Aa against DOBV-Af and Saaremaa-Aa in most areas of the alignment, the tree topology could not be resolved in the first 225 nt of the ORF, where the Esl/8xxAa – Kurkino-Aa subtree forms a multifurcation with DOBV-Af and Saaremaa-Aa. In the area from nt 400 - 600 a change in the topology was found where DOBV-Af branched off at the root of the Esl/8xxAa – Kurkino-Aa subtree. However, one should note that the support (64 %) for this was not very high. In contrast to the loss of support for the Esl/8xxAa – Kurkino-Aa monophyly in the bootscan in the area from 730-1080 nt, the phylogenetic tree maintains this monophyly with high support of 82%.

This discrepancy between bootscanning and TREE-PUZZLE as well as the low overall support for the Esl/8xxAa – Kurkino-Aa monophyly in the bootscanning, can be explained as follows. While the ML quartet puzzling analysis and the resulting PUZZLE support values

made use of the full data set, the bootscanning is based on bootstrapping from windows of a certain size, here 200 nt. Bootstrapping draws pseudosamples with replacement from the alignment columns by also discarding alignment columns. The low divergence of the S segments of 29.9 - 51.7 % constant sites in the subalignments (for comparison, M segments have 18.4 - 41.4 % constant sites) can lead to a loss of information in the bootstrapping which results in less resolved trees and subsequently in low values in the bootscanning. For this reason the S segment ORFs are less suitable for bootscanning analysis. However, it is still a good tool to find split points of the alignment for further phylogenetic analysis.

In the analysis of the M segment the resulting picture gave more clear results. First, the support values in the bootscanning reached up to 100% (cf. above and Figure 10). Furthermore the main DOBV topology divided into Esl/862Aa with Saa/160v on the one hand and Slo/Af with Esl/400Af on the other yielding high support (92-100%). In two areas of the ORF this topology changed bringing together Saa/160v and the DOBV-Af group. While this change in grouping of M segment regions had moderate support (62%) in the area 1971-2211 nt, the support in the area 810-1059 nt with 81% was very high.

Homologous recombination events could explain the creation of this significant nucleotide sequence exchange. However, this issue needs to be further evaluated, taking into account biological properties of M segment gene products which are currently not very well characterised.

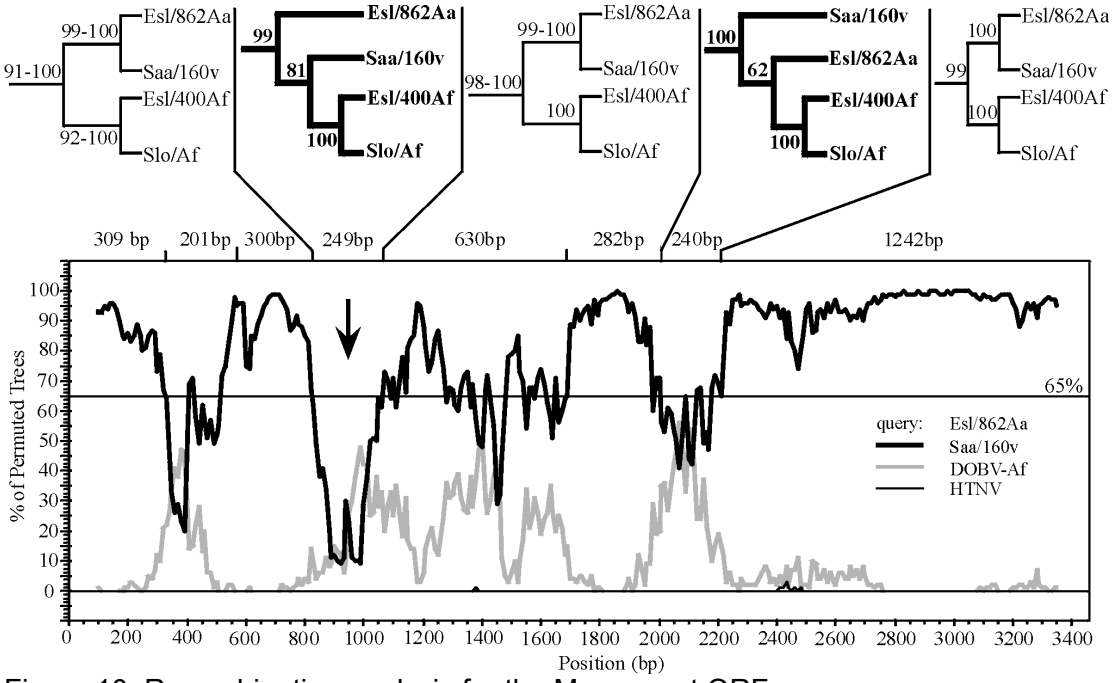

Figure 10: Recombination analysis for the M segment ORFs.
The lower part shows the bootscan diagram using Esl/862Aa as query, and the other viruses grouped into DOBV-Af, Saaremaa-Aa (Saa/160v), and HTNV. The upper part shows the ML DOBV subtrees using TREE-PUZZLE from the subalignments split at a 65% threshold in the bootscan diagram. At the branches the PUZZLE support values for the subtrees are given. Where neighbouring subalignment resulted in the same tree topology only one with ranges of support values is shown.

3.2.5 Identification of putative host-specific differences in the virus-coded N and GPC

When directly comparing the N protein sequences of Esl/856Aa and Esl/862Aa on one hand and Esl/400Af on the other we have found a total of 9 aa exchanges, 8 conservative and 1 non-conservative ones according to Dayhoff exchange groups (Dayhoff *et al*., 1978). Including the Russian Kurkino-Aa strains and Slo/Af in this comparison reduced the putatively host-specific genetic differences to 7 aa exchanges (Figure 11A). Furthermore, when including Saaremaa-Aa in the DOBV-Aa group, only 3 aa differences (out of 7) have been found between the DOBV-Aa strains on the one hand and the DOBV-Af strains on the other (S13N, I295L, and R356K) which may represent host-specific residues (Figure 11A). According to Dayhoff exchange groups, all three aa exchanges represent conservative exchanges. The Saaremaa-Aa S segment has been included into this analysis despite our finding that it is phylogenetically related to the DOBV-Af S segment (see above). Nevertheless, the virus underwent its (at least rather recent) evolution in *A. agrarius* and should have accumulated mutations which did allow replication in this host.

Position 295 is located in the highly variable part of N protein (Plyusnin *et al*., 1996) whereas the other two exchanges (pos. 13, 356) had occurred in rather conserved regions of N (Arikawa *et al*., 1990). Position 13 lies in the major immunodominant region of N proteins from other hantavirus species which includes B- and T-cell epitopes (de Carvalho *et al*., 2001; Gott *et al*., 1997; Jenison *et al*., 1994; Lundkvist *et al*., 2002b; Van Epps *et al*., 1999).

The comparison with N protein aa sequences of other hantavirus species at position 13 showed amino acids always different from DOBV-Aa and DOBV-Af strains. At the other positions (aa 295 and 356) the same aa residues as in one of the DOBV lineages could be found. (data not shown). At aa 356 of all hantaviral N proteins the basic aa residues R or K have been found to be conserved which might be caused by the nucleic acid binding function of the C-terminal region previously shown for PUUV-N protein (Gott *et al*., 1993).

In a direct comparison of glycoprotein (G1/G2) aa sequences of strains Esl/862Aa and Esl/400Af we found 66 aa differences. When including the only available complete M sequences of Saa/160v and Slo/Af in the analysis, 38 aa exchanges (27 conservative and 11 non-conservative ones) remained which exist between the two DOBV-Aa strains on the one side and the two DOBV-Af strains on the other (Figure 11B). Moreover, when including GPC aa sequences of other hantaviruses, four positions (at aa 14, 15, 230, 335) were found, where every hantavirus species has a specific aa residue. The host specificity of aa 14 and 15 may be due to their functional role at the C-terminal end of the signal peptide of the GPC.

Sequence determination of more DOBV strains from different regions of Europe should enable a further specification of host-dependent genetic differences within the virus species.

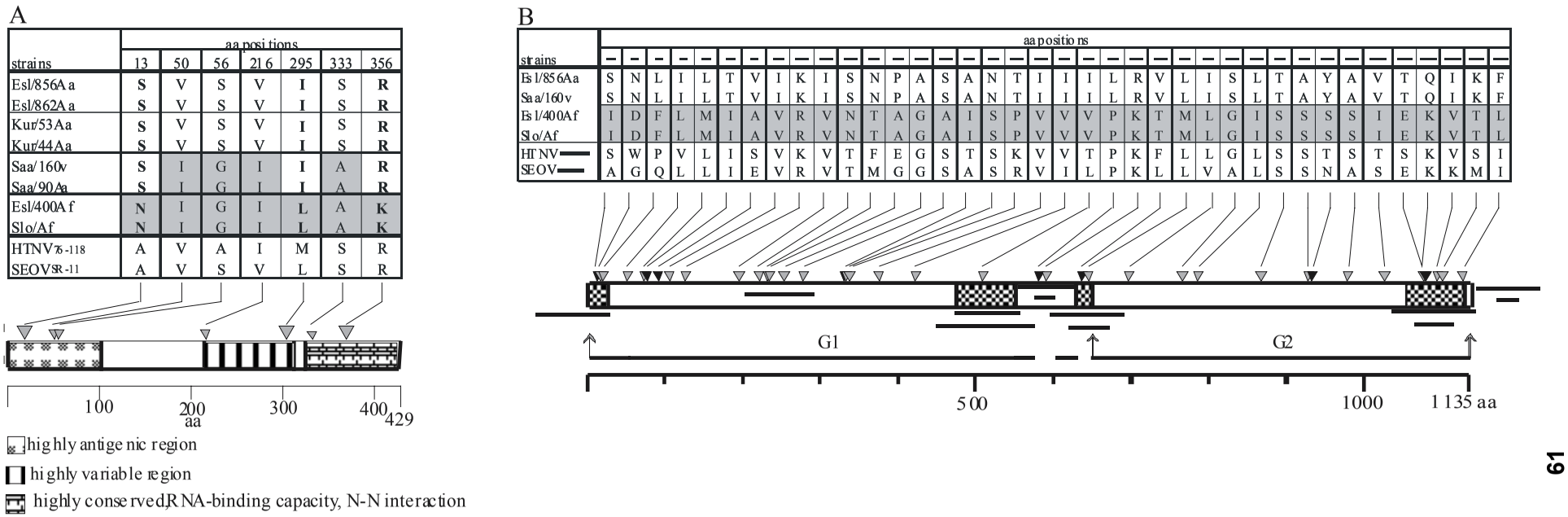

Figure 11: Putative host specific amino acid exchanges in the N protein (Figure 11A) and the glycoproteins (Figure 11B) of DOBV strains from *A. flavicollis* vs. *A. agrarius* hosts.

Upper parts show aa residues at sites where DOBV-Aa and DOBV-Af differ. Gray background indicates DOBV-Af-like residues.
Lower parts: Triangles indicate putative host specific aa exchanges. Black triangles indicate non-conservative, gray triangles conservative aa exchanges (according to Dayhoff exchange groups).
Figure 11A: Large triangles represent aa exchanges where Saaremaa-Aa strains exhibit the same amino acid as other DOBV-Aa strains. Small triangles symbolise exchanges, where Saaremaa strains resemble the DOBV-Af strains. Typical hantavirus N protein regions (Arikawa *et al.*, 1990; Plyusnin *et al.*, 1996) are marked. Figure 11B: The positions of the G1 and G2 glycoproteins as well as other functionally important domains (Plyusnin *et al.*, 1996) are shown.

3.3 Genetic diversity of DOBV on single geographical locus

3.3.1 DOBV in Rozhanovce locality, Eastern Slovakia

As a part of long-term epizootiologic survey, small rodents were trapped on Rozhanovce locality in Eastern Slovakia during three trapping nights in September and October of 2001. This locality was selected on the basis of reported human cases of HFRS. The trapping area was situated in a pheasants breeding station.

57 rodents were trapped. Identified rodent species included *A. agrarius* (n=42), *A. flavicollis* (n=9) and *C. glareolus* (n=6). Antibodies reactive with DOBV antigen were found only in *A. agrarius* rodents, however, the antibody prevalence was very high. 11 out of 42 (26.2%) mice were found sero- and RT PCR-positive. Six of them, designed Esl/33Aa, Esl/193Aa, Esl/194Aa, Esl/197Aa, Esl/200Aa, and Esl/201Aa, were selected for cloning and sequencing of partial S (position 357-955 nt, 559 nt in length when excluding the primer sequences) and M (1674-1990 nt, 271 nt when excluding the primer sequences) segments.

In addition, we have determined the complete S segment nucleotide sequence of strain Esl/33Aa from Rozhanovce and of strains Esl/29Aa and Esl/81Aa detected in neighbouring localities Sebastovce and Botany, respectively. Esl/29Aa and Esl/81Aa S segments were found to be 7 nt shorter (1697 nt) than sequence of Esl/862Aa, due to an 8 nt deletion and an 1 nt insertion located in 3' NCR. Esl/33Aa harboured one additional nt deletion, thus reaching 1696 nt in length.

Sequence comparison of these six DOBV sequences originating from Rozhanovce with other DOBV sequences showed interesting results. From S segment sequence identity matrix (Table 9) is obvious that these six sequences originating from one trapping site represent two distinct groups of sequences (Esl/33Aa, Esl/193Aa, Esl/200Aa and Esl/201Aa on one hand, Esl/194Aa and Esl/197Aa on the other hand) exhibiting relatively high sequence diversity of 5.4 – 5.7% while the sequence identity within these groups is reaching 98.6 – 100%.

Similar relationships could be deduced from the comparative analysis of the partial M segment sequences (Table 10). However, the overall diversity of studied sequences is lower, most likely because of short sequenced fragment. Interestingly, strains Esl/194Aa and Esl/197Aa exhibited higher similarity to previously identified DOBV strains from Eastern Slovakia, Esl/856Aa and Esl/862Aa, than to the other Rozhanovce derived strains. However, both groups represent unique sequences. Hence, the possibility that only one group represents real DOBV sequences from Rozhanovce and the other one is only a laboratory contamination by some other DOBV strains previously identified in our laboratory could be excluded.

Maximum Likelihood phylogenetic analysis of S segment partial sequences (Figure 12) confirmed these observations. The sequences originating from rodents captured on a single trapping place do not form a monophyletic group. Whereas the strains Esl/33Aa, Esl/193Aa, Esl/200Aa and Esl/201Aa form one group and cluster with strains from neighbouring trapping localities, the strains Esl/194Aa and Esl/197Aa form well supported group with strains Esl/856Aa and Esl/862Aa originating also from Eastern Slovakia but from another locality (Novy Ruskov). We could see the same branching topology also on phylogenetic tree based on partial M segment sequences (data not shown).

These interesting observations do not agree with the geographical clustering, which is usually observed in hantaviruses of the same genotype and originating from the same rodent host. An explanation, which is in agreement with the virus-host coevolution concept (Plyusnin and Morzunov, 2001), consists in the simultaneous presence of two phylogenetically distinct subpopulations of *A. agrarius* in Rozhanovce locality, carrying distinct DOBV strains.

Table 9: S segment nucleotide percent identity of Rozhanovce-originating and other DOBV strains

Strain*		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.
1.	Esl/33Aa	-	99.2	98.9	99.2	99.1	98.7	94.4	94.4	94.2	94.2	90.8	90.6	85.1	85.1	84.2	84.6
2.	Esl/193Aa		-	99.6	100	99.4	99.1	94.2	94.2	93.7	93.7	90.5	90.3	85.1	85.1	83.8	84.6
3.	Esl/200Aa			-	99.6	99.1	98.7	93.9	93.9	93.3	93.3	90.1	89.9	84.7	84.7	83.8	84.6
4.	Esl/201Aa				-	99.4	99.1	94.2	94.2	93.7	93.7	90.5	90.3	85.1	85.1	83.8	84.6
5.	Esl/29Aa					-	99.2	93.9	93.9	93.3	93.3	90.5	90.3	85.1	85.1	84.2	84.9
6.	Esl/81Aa						-	93.5	93.5	93.0	93.0	90.6	90.5	85.3	85.3	84.4	85.1
7.	Esl/194Aa							-	100	99.1	99.1	89.9	89.8	86.7	87.1	85.3	85.8
8.	Esl/197Aa								-	99.1	99.1	89.9	89.8	86.7	87.1	85.3	85.8
9.	Esl/856Aa									-	100	89.8	89.6	86.5	87.2	84.4	85.6
10.	Esl/862Aa										-	89.8	89.6	86.5	87.2	84.4	85.6
11.	Kur/53Aa											-	99.8	87.4	87.1	87.2	87.2
12.	Kur/44Aa												-	87.2	86.9	87.1	87.1
13.	Saa/160V													-	98.2	87.4	88.0
14.	Saa/90Aa														-	86.9	88.0
15.	Esl/400Af															-	96.2
16.	Slo/Af																-

An S segment partial sequences (377-935 nt) were analysed. The identity values were calculated using Clustal method. The percentage differences for nucleotide sequences are presented. *For strain abbreviations, see Table 5.

Table 10: M segment nucleotide percent identity of Rozhanovce-associated and other DOBV strains

Strain*		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1.	Esl/33Aa	-	99.6	99.6	99.6	98.1	98.1	98.5	88.1	83.0	81.9
2.	Esl/193Aa		-	100	100	98.5	98.5	98.8	87.8	83.3	81.5
3.	Esl/200Aa			-	100	98.5	98.5	98.8	87.8	83.3	81.5
4.	Esl/201Aa				-	98.5	98.5	98.8	87.8	83.3	81.5
5.	Esl/194Aa					-	100	99.6	88.5	82.6	80.8
6.	Esl/197Aa						-	99.6	88.5	82.6	80.8
7.	Esl/862Aa							-	88.5	82.6	80.8
8.	Saa/160v								-	83.3	83.0
9.	Esl/400Af									-	94.0
10.	Slo/Af										-

An M segment partial sequence (1700-1970 nt) was analysed. The identity values were calculated using Clustal method. The percentage differences for nucleotide sequences are presented. *For strain abbreviations, see Table 5.

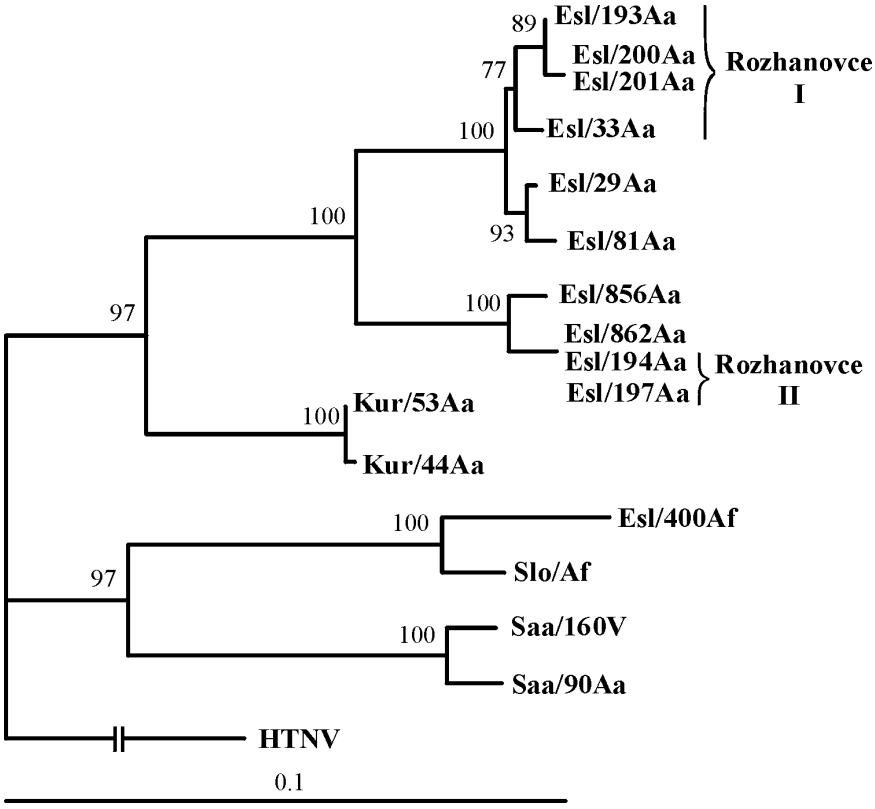

Figure 12: Phylogenetic ML tree based on the partial S segment nt sequences (377-935 nt) of Rozhanovce-originating and other DOBV strains, computed with TREE-PUZZLE.

Strains with identical sequence are represented by single branch. HTNV strain 76-118 was used as an outgroup. For graphical presentation, to obtain a better resolution of the picture in the analysed Rozhanovce clades, the length of the longest branch indicated by two vertical lines (HTNV as an outgroup) was shortened by 0.1 of evolutionary distance. For strain name abbreviations and sequence references see Table 5.

3.3.2 Rodent genetics

In order to find out whether there are two distinct subpopulations of *A. agrarius* in Rozhanovce locality, we carried out a sequence analysis of rodent genetic material using two phylogenetic mitochondrial markers, 12S rRNA gene (in collaboration with Dr. Plötner, Museum of Natural History, Berlin, Germany) and control region of mitochondrial DNA (D-loop). For DNA extraction and PCR amplification, we used material from four DOBV-positive individuals from Rozhanovce locality (Apa194/01 and Apa197/01, which carry DOBV Rozhanovce group II; Apa200/01 and Apa201/01, which carry DOBV Rozhanovce group I), and individuals of *A. agrarius* and *A. flavicollis* from other three selected localities in Slovakia (Table 11, Figure 13).

We have established the nucleotide sequence of 12S rRNA genetic marker (372 nt) of 10 *A. agrarius* and six *A. flavicollis* mice captured in Slovakia. The sequence identity matrix and multiple sequence alignment of polymorphic sites are shown in Table 12 and Table 13, respectively. There was a significant diversity of 7.2 –7.8% between the *A. agrarius* and *A. flavicollis* sequences. However, the variability within the species is so low that there was not enough phylogenetic information to construct a reasonable phylogenetic tree.

In a second attempt to study the evolutionary history of *A. agrarius* population from Slovakia, we have sequenced a part of mitochondrial DNA (1167 nt) including D-loop of all *A. agrarius* animals mentioned above. Despite relatively long sequences studied, we could find only few variable sites (n=13) in the sequence alignment (Table 14) and the sequence identities were reaching 99.3 – 100% (Table 15).

Altogether, the analysis of both 12S rRNA and D-loop genetic markers revealed lack of sequence variability within the *A. agrarius* mice captured in Eastern Slovakia. This suggests that according to these markers all these individuals represent a single population. Moreover, the analysis of 12S rRNA gene showed that this marker is suitable for distinguishing at least *A. agrarius* and *A. flavicollis* species and also confirmed that the individuals used in this study were previously well identified according to the morphologic criteria used.

Table 11: *Apodemus* mice used for amplification and sequencing of mitochondrial markers 12S rRNA gene and D-loop

Sample		Locality	Geographic location
Apodemus agrarius			
1.	Apa194/01	Rozhanovce	Eastern Slovakia
2.	Apa197/01	Rozhanovce	Eastern Slovakia
3.	Apa200/01	Rozhanovce	Eastern Slovakia
4.	Apa201/01	Rozhanovce	Eastern Slovakia
5.	Apa24/01	Sebastovce	Eastern Slovakia
6.	Apa25/01	Sebastovce	Eastern Slovakia
7.	Apa95/99	Krasny Brod	Eastern Slovakia
8.	Apa99/99	Krasny Brod	Eastern Slovakia
9.	Apa64/01	Botany	Eastern Slovakia
10.	Apa65/01	Botany	Eastern Slovakia
A. flavicollis			
11.	Apf95/01	Zahorska Ves	Western Slovakia
12.	Apf96/01	Zahorska Ves	Western Slovakia
13.	Apf46/01	Sebastovce	Eastern Slovakia
14.	Apf47/01	Sebastovce	Eastern Slovakia
15.	Apf110/99	Krasny Brod	Eastern Slovakia
16.	Apf111/99	Krasny Brod	Eastern Slovakia

The sample names consist of abbreviation of rodent species (Apa stands for *A. agrarius,* Apf for *A. flavicollis*) / year of trapping. For better geographic location of trapping sites, see Figure 13.

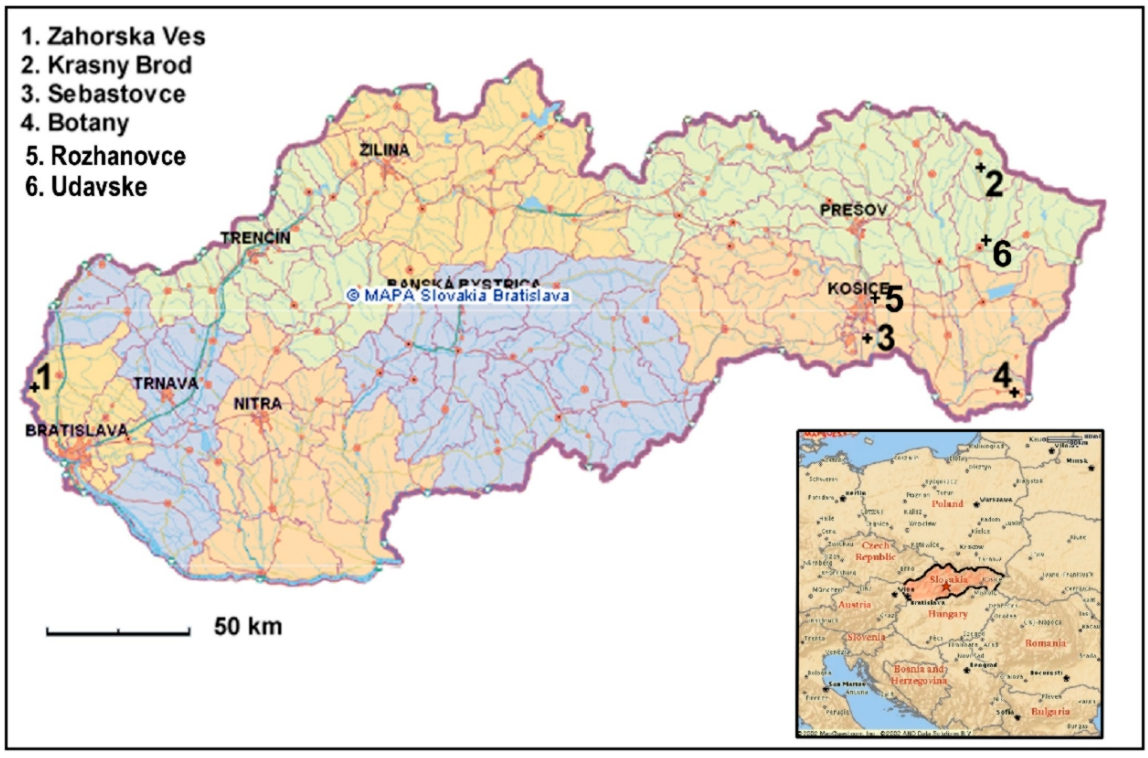

Figure 13: Map of Slovakia with trapping localities for virus and rodent genetics studies.

Table 12: 12s rRNA gene nucleotide sequence percent identity of *Apodemus* mice trapped on selected localities of Slovakia

Sample		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.
1.	Apa194/01	-	99.7	100	100	100	100	100	99.7	100	100	92.7	92.7	92.7	92.7	92.7	92.4
2.	Apa197/01		-	99.7	99.7	99.7	99.7	99.7	99.4	99.7	99.7	92.4	92.4	92.4	92.4	92.4	92.2
3.	Apa200/01			-	100	100	100	100	99.7	100	100	92.7	92.7	92.7	92.7	92.7	92.4
4.	Apa201/01				-	100	100	100	99.7	100	100	92.7	92.7	92.7	92.7	92.7	92.4
5.	Apa24/01					-	100	100	99.7	100	100	92.7	92.7	92.7	92.7	92.7	92.4
6.	Apa25/01						-	100	99.7	100	100	92.7	92.7	92.7	92.7	92.7	92.4
7.	Apa95/99							-	99.7	100	100	92.7	92.7	92.7	92.7	92.7	92.4
8.	Apa99/99								-	99.7	99.7	92.4	92.4	92.4	92.4	92.4	92.2
9.	Apa64/01									-	100	92.7	92.7	92.7	92.7	92.7	92.4
10.	Apa65/01										-	92.7	92.7	92.7	92.7	92.7	92.4
11.	Apf95/01											-	100	99.4	100	100	99.7
12.	Apf96/01												-	99.4	100	100	99.7
13.	Apf46/01													-	99.4	99.4	99.7
14.	Apf47/01														-	100	99.7
15.	Apf110/99															-	99.7
16.	Apf111/99																-

12S rRNA mitochondrial gene sequences (372 nt) were analysed. The identity values were calculated using Clustal method. The percentage differences for nucleotide sequences are presented.

Table 13: Multiple alignment of a partial 12S rRNA gene sequence (372 nt) from *Apodemus* sp. mice captured in Slovakia; only polymorphic sites are shown.

	polymorphic sites																													
	7	8	11	18	43	44	83	111	118	139	142	143	187	188	204	205	206	211	269	270	271	281	282	287	288	301	309	337	349	356
Apa194/01	T	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	A	T	T	A	G	T
Apa197/01	T	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	G	T	T	A	G	T
Apa200/01	T	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	A	T	T	A	G	T
Apa201/01	T	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	A	T	T	A	G	T
Apa24/01	T	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	A	T	T	A	G	T
Apa25/01	T	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	A	T	T	A	G	T
Apa64/01	T	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	A	T	T	A	G	T
Apa65/01	C	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	A	T	T	A	G	T
Apa95/99	T	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	A	T	T	A	G	T
Apa99/99	T	G	C	T	T	A	C	A	C	T	-	T	T	A	T	T	T	A	A	T	T	T	T	A	A	T	T	A	G	T
Apf95/01	T	A	T	C	C	T	T	C	A	C	A	T	A	T	C	A	-	T	T	A	C	C	-	T	A	C	C	G	A	A
Apf96/01	T	A	T	C	C	T	T	C	A	C	A	T	A	T	C	A	-	T	T	A	C	C	-	T	A	C	C	G	A	A
Apf46/01	T	A	T	C	C	T	T	C	A	C	A	C	A	T	C	A	-	T	T	A	C	C	-	T	A	C	C	G	G	A
Apf47/99	T	A	T	C	C	T	T	C	A	C	A	T	A	T	C	A	-	T	T	A	C	C	-	T	A	C	C	G	A	A
Apf110/99	T	A	T	C	C	T	T	C	A	C	A	T	A	T	C	A	-	T	T	A	C	C	-	T	A	C	C	G	A	A
Apf111/99	T	A	T	C	C	T	T	C	A	C	A	C	A	T	C	A	-	T	T	A	C	C	-	T	A	C	C	G	A	A

Table 14: Multiple alignment of mitochondrial DNA (1167 nt) including D-loop of *A. agrarius* mice captured in Slovakia; only polymorphic sites are shown.

	polymorphic sites												
	22	214	240	271	326	719	776	781	788	789	816	841	1085
Apa194/01	A	-	G	C	C	G	T	G	T	T	A	A	A
Apa197/01	A	T	A	T	T	G	T	A	A	C	A	A	G
Apa200/01	-	-	A	C	C	T	C	A	A	C	A	T	A
Apa201/01	-	-	A	C	C	G	T	A	A	C	A	T	A
Apa24/01	A	-	A	C	C	G	T	A	A	C	A	A	A
Apa25/01	A	-	A	C	C	G	T	A	G	C	A	A	A
Apa95/99	A	-	A	C	C	G	T	G	A	C	G	A	A
Apa99/99	A	-	A	C	C	G	T	G	A	C	G	A	A
Apa64/01	A	-	A	C	C	G	T	A	A	C	A	A	A
Apa65/01	A	-	A	C	C	G	T	A	A	C	A	A	A

Table 15: D-loop nucleotide sequence percent identity of *A. agrarius* mice trapped on selected localities of Slovakia

Sample		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1.	Apa194/01	-	99.3	99.3	99.4	99.6	99.6	99.6	99.6	99.6	99.6
2.	Apa197/01		-	99.3	99.4	99.6	99.5	99.4	99.4	99.6	99.6
3.	Apa200/01			-	99.8	99.6	99.5	99.4	99.4	99.6	99.6
4.	Apa201/01				-	99.8	99.7	99.6	99.6	99.8	99.8
5.	Apa24/01					-	99.9	99.8	99.8	100	100
6.	Apa25/01						-	99.7	99.7	99.9	99.9
7.	Apa95/99							-	100	99.8	99.8
8.	Apa99/99								-	99.8	99.8
9.	Apa64/01									-	100
10.	Apa65/01										-

3.4 Isolation of DOBV from *A. agrarius* captured in East Slovakia

3.4.1 Virus isolation and titration

In order to obtain a cell culture isolate of DOBV representing the Central European DOBV-Aa genetic lineage, tissues of sero- and RT-PCR-positive *A. agrarius* were used for virus isolation attempts. 10% suspensions of homogenised tissues (liver of Esl/34Aa , lung of Esl/35Aa) were inoculated onto confluent Vero E6 cells in three 25 cm^2 flasks each. Cells were passaged at days 14, 39 and 47 post infection (p.i.). After the third passage at day 47 p.i., in the flasks of both samples some hantavirus antigen-positive cells were found by IFA using a DOBV-specific human convalescent serum. After the fourth passage (day 59), most cells were found to be positive. One isolate, originating from the field strain DOBV/East Slovakia/34AaV/2001 was then further passaged and designated Slovakia (short name SK/Aa, where SK stands for Slovakia and Aa indicates for better clarity the natural host).

Cell culture supernatants harvested from 3^{rd} –5^{th} passages were titrated using a focus assay. Whereas the titers of third and fourth passage supernatants were extremely low (< 10^3 focus forming units, FFU/ml), the fifth passage supernatant had markedly higher titer (4.5 x 10^5 FFU/ml). A cell culture supernatant harvested from fifth passage (day 51) was therefore regarded as a first virus stock (stock A).

Since hantaviruses do not naturally form plaques in Vero E6 cell culture allowing plaque purification of the virus we had to follow an alternative approach based on limiting dilution to obtain a homogenous virus stock., Ten wells of six-well plates were inoculated with the stock A diluted to 0.5 FFU/well. After ten days incubation, supernatant samples were collected and the cells were examined using the chemiluminiscent focus assay. Four out of ten wells were found to be antigen-positive. The supernatant from one of the positive wells was then used to infect new Vero E6 cells and produce new viral stock (B) for further analyses. Assuming that the positive wells were infected with a single virus particle, we obtained a homogenous virus stock equivalent to a plaque-purified virus. The titre of stock B was determined to be 5.0 x 10^5 FFU/ml.

3.4.2 Sequence analysis of SK/Aa genomic segments

The complete S segment sequence of the new isolate SK/Aa was determined to be 1697 nt in length containing a single ORF (nt 36 - 1,325) for the viral N protein which encodes a putative protein of 429 aa length. In addition, the complete S segment nucleotide sequences of wild strains Esl/29Aa and Esl/81Aa were obtained from the lung tissues of *A. agrarius* animals and were determined to be of the same length and coding structure.

As expected, the sequences of Esl/29Aa and Esl/81Aa were found to be very similar to the new isolate (98.9 - 99.0% nt and 99.7% aa identities). When compared with other DOBV sequences, previously determined DOBV-Aa sequences from East Slovakia (Esl/856Aa and Esl/862Aa) showed the highest similarity (93.3 nt and 99.3% aa identity). However, the S segment of the new isolate was found to be 7 nt shorter than the sequences of Esl/856Aa and Esl/862Aa strains, due to an 8 nt deletion and an 1 nt insertion located in the 3' NCR. The most dissimilar DOBV strain on nt level, Esl/400Af (84.4% nt identity), is from the same geographical region of Slovakia but belongs to the DOBV-Af lineage. On the deduced N protein aa sequence level, the Saa/160V isolate showed the highest diversity to SK/Aa, even higher than the virus isolates of DOBV-Af lineage, Slo/Af and AP/Af (Table 16, upper part). Five cysteine residues were found in the N protein aa sequence (at aa positions 203, 244, 293, 315 and 319), all of them are highly conserved and were found in all N protein sequences analysed here (Table 16).

The total M segment nucleotide sequence of SK/Aa was found to be 3,643 nt in length. It contains a single ORF (nt 41 to 3445) encoding the putative GPC of 1,135 aa in length. Table 16 (lower part) shows the degree of M segment nucleotide and deduced amino acid sequence identity between the DOBV isolates. The DOBV-Af isolate Slo/Af was found to be the most dissimilar (only 81.5% nt and 93.4% aa identity) what could explain the distinct serological behaviour of SK/Aa and Slo/Af in cFRNT (see below). The GPC aa sequence of the of SK/Aa showed the typical sequence motifs of hantavirus glycoproteins described above for Esl/862Aa and Esl/400Af.

In addition, a partial L segment sequence of 541 nt length (nt position 109-649 according to the co-ordinates in the L segment of DOBV AP/Af, GenBank accession number AJ410617) from the SK/Aa isolate was determined. The comparison with the only available complete DOBV L segment sequences showed relatively low nt sequence identities of 86.3 and 85.0% to the Saa/160V and AP/Af strains, respectively. In contrast, percent-identity values of the corresponding amino acids (180 aa, aa positions 25-204) were very high, reaching 97.7 % towards both Saa/160V and AP/Af strains. This shows that most of the nucleotide exchanges represented silent mutations.

It should be mentioned that the S, M, and L segment sequences determined for SK/Aa did not encompass any known sequence from any virus strain handled in our laboratory. This clearly shows that no contamination problem had occurred.

3.4.3 Phylogenetic analysis

To avoid the phylogenetic analysis being disturbed by conflicting phylogenetic signal due to potential recombination events, both SK/Aa complete S and M segment sequences

were first screened by similarity plots and bootscanning. No significant recombination signals, which could disturb construction of the phylogenetic trees, were found (data not shown).

Complete coding sequences, allowing unambiguous aligning on amino acid level, were used to construct Maximum likelihood trees assuming Tamura-Nei evolutionary model with gamma distributed rate heterogeneity over sites. In the S segment ML phylogenetic tree (Figure 14a), the new isolate formed a well-supported monophyletic group with all DOBV-Aa strains except Saaremaa strains which clustered together with the DOBV-Af strains (see below). As expected, all other Slovakian strains were the nearest relatives of the SK/Aa. The new strains SK/Aa, Esl/29Aa and Esl/81Aa formed a sister group to the previously characterised strains Esl/856Aa and Esl/862Aa. *A. flavicollis*-derived strains from Slovakia (Esl/400Af), Greece (AP/Af) and Slovenia (Slo/Af) formed the second cluster sharing the common ancestor with the Saaremaa strains.

In the M segment ML tree (Figure 14b), SK/Aa again clustered with the Esl/862Aa strain. However, in contrast to the S segment analysis, SK/Aa shared a common ancestor also with the Saa/160Aa strain from Estonia. The strains Esl/400Af, AP/Af, and Slo/Af formed again a strongly supported monophyletic group representing the DOBV-Af lineage.

Phylogenetic analysis of partial L segment sequences (541 nt) showed results consistent with the S segment analysis. AP/Af strain clustered with the Saa/160V strain while SK/Aa formed an outgroup to this well-supported cluster (Figure 14a). When using only 374 nt long sequences (nt position 157-530), the available partial sequence of the original DOBV isolate, Slo/Af, could be included in the alignment. Also in this case, SK/Aa was the most ancestral sequence and Saa/160V formed a well supported monophyletic group with the AP/Af and Slo/Af strains (data not shown).

To enable a direct comparison of the phylogenetic trees based on S, M, and L segment sequences, the S and M segment phylogenetic analysis was repeated only with sequences of those strains which were also used for construction of the L segment phylogenetic tree. Nevertheless, clustering of the Saa/160V strain with the DOBV-Af strains in S and L segment phylogeny (Figure 15a, b) but with DOBV-Aa strains in M segment phylogeny (Figure 15c) remained unchanged.

Table 16: Complete S and M segment nucleotide and amino acid sequence identities of SK/Aa with other DOBV, HTNV and SEOV isolates.

strains	% identity with virus strain:					
	SK/Aa	Saa/160V	Slo/Af	AP/Af	HTNV 76-11	SEOV SR11
S segment / N protein						
SK/Aa	-	86.2	85.7	85.2	70.8	68.1
Saa/160V	96.9	-	88.3	87.3	69.7	66.2
Slo/Af	97.6	97.4	-	96.6	70.7	67.4
AP/Af	98.1	97.4	99.5	-	70.5	67.6
HTNV 76-11	83.6	82.5	82.5	82.9	-	67.5
SEOV SR11	81.1	79.9	79.9	80.4	82.2	-
M segment / GPC						
SK/Aa	-	86.8	81.5	81.5	69.0	69.6
Saa/160V	95.7	-	81.3	81.5	69.6	69.5
Slo/Af	93.4	94.1	-	93.4	69.1	69.4
AP/Af	93.5	94.1	98.5	-	69.7	69.7
HTNV 76-11	77.0	76.9	77.1	77.3	-	71.3
SEOV SR11	76.7	76.4	77.2	77.1	76.8	-

The percentage differences for nucleotide (above the diagonal) and amino acid (below the diagonal) sequences are presented.

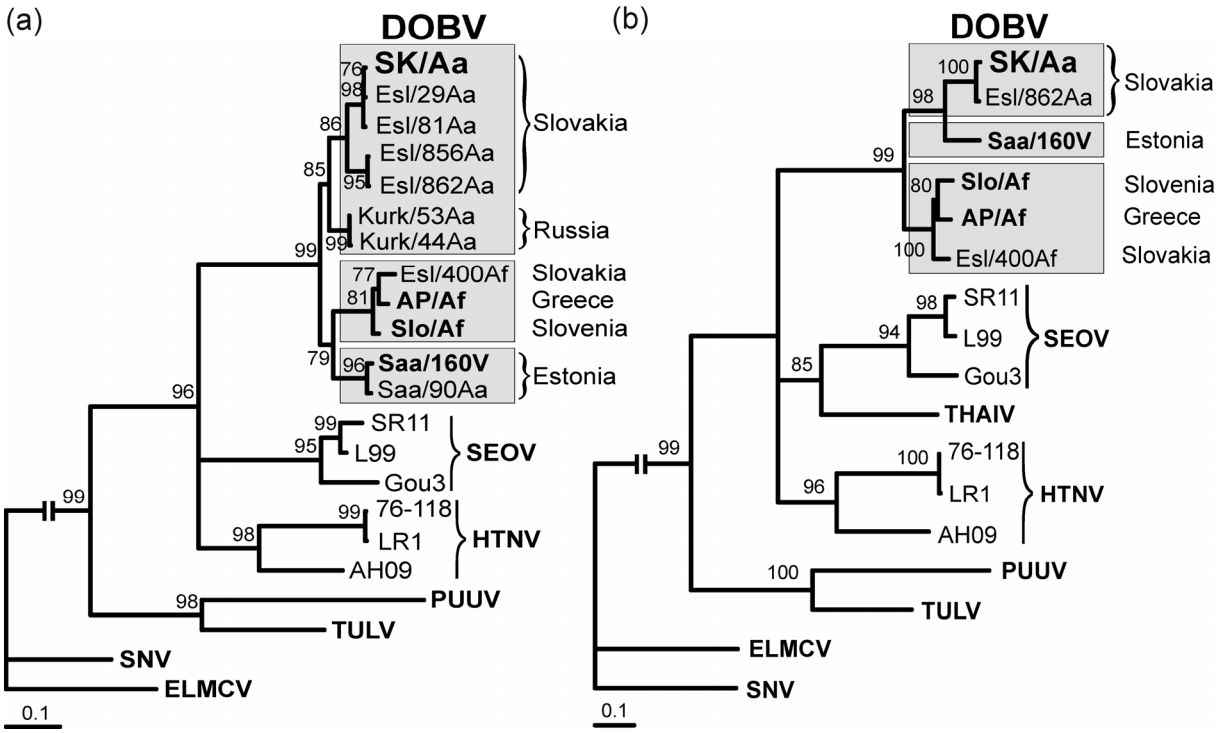

Figure 14: Maximum likelihood phylogenetic trees of DOBV strains based on (a) complete S and (b) complete M segment ORF nucleotide sequences (corresponding to S segment nt sequence position 36 - 1,325 and M segment nt position 41 to 3,445 of SK/Aa, respectively).

DOBV isolates are in bold. Three proposed DOBV lineages are marked by grey boxes. For abbreviations and accession numbers, see the Material and methods section. The trees were computed with the TREE-PUZZLE. The values at the tree branches are the PUZZLE support values.

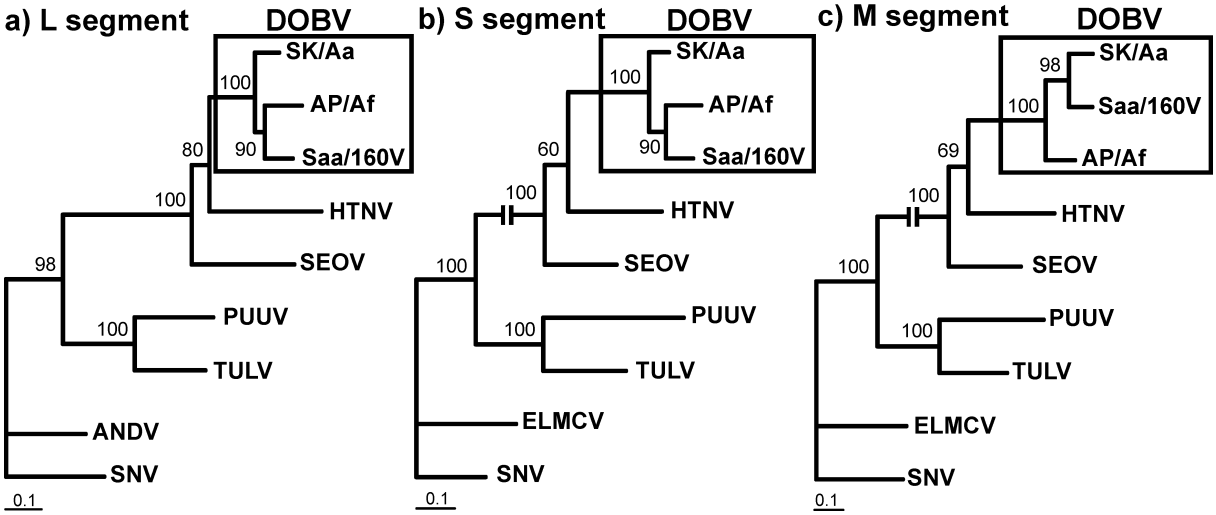

Figure 15: Maximum likelihood phylogenetic trees of DOBV virus isolates based on (a) partial (541 nt, position 109 - 649) L segment sequences, (b) complete S ORF, and (c) complete M ORF nucleotide sequences (corresponding to S segment nt position 36 - 1,325 and M segment nt position 41 to 3,445, respectively).

The trees were computed with the TREE-PUZZLE package. The values at the tree branches are the PUZZLE support values.

3.4.4 *In vitro* evolution of virus during passaging

Isolation of hantaviruses on Vero E6 cells is a tedious process including blind passages during several weeks. It is believed that during this time, the virus has to undergo adaptation to cell culture resulting in changes of nucleotide and amino acid sequences. We were interested to determine whether the nucleotide sequence of the S segment underwent changes during the virus isolation procedure in cell culture. For this purpose we compared the sequences of the original hantaviral RNA in the rodent tissue (Esl/34Aa) and of the cell-culture adapted isolate SK/Aa. In the complete S segment we found one single nt exchange G (rodent tissue) → A (isolate) at position 162 resulting in an amino acid exchange Ala (GCA) → Thr (ACA) at aa position 43 of viral N protein. Only this single mutation was found in the S segment sequence even after 11 passages of virus growth. Interestingly, amino acid Ala (GCA) at position 43 found in Esl/34Aa is also present in all other DOBV N protein sequences including the Vero E6-adapted isolates Slo/Af, Saa/160V and AP/Af. On the other hand, Thr at this position is harbored by N proteins of many Hantaan virus (HTNV) and Seoul virus (SEOV) strains and most of the other hantaviruses.

3.4.5 Antigenic characterisation of the DOBV-SK/Aa isolate

First antigenic characterisation was performed by IFA with a panel of eight mAbs directed against HTNV, SEOV and ANDV N protein. The obtained pattern for the SK/Aa and Slo/Af isolates was identical (Table 17, upper part). Both isolates reacted with Eco2, E5/G6, C116D11 and 2E2 mAbs (all raised against HTNV) while both did not react with B5D9, C24B4 (both against HTNV), R31 (against SEOV), and 5C2/E10 (against ANDV). In the cases where the respective epitope locations in the N protein are known a comparison of the SK/Aa and Slo/Af sequences at these positions showed no or only a few aa exchanges which could all be considered as conservative according to Dayhoff *et al*. (1978) (Table 17, lower part).

Nine convalescent sera of HFRS patients from Germany and Slovakia were used to compare the neutralising antibody titers against the new isolate SK/Aa as a representative of the DOBV-Aa lineage and the prototype strain Slo/Af from the DOBV-Af lineage. All sera from German HFRS patients have been serotyped as anti-DOBV specific by FRNT (Sibold *et al*., 2001; chapter 3.6).

Four patient's sera showed four- and one serum sixteen-fold higher reciprocal titer to SK/Aa than to Slo/Aa. In contrast, two sera exhibited four-fold higher reciprocal titer to Slo/af strain. Two sera reacted at equal end point titers with both viruses (Table 18).

Table 17: Reactivity of DOBV isolates Slovakia and Slovenia in IFA with the panel of anti-N protein monoclonal antibodies.

Virus	Monoclonal antibody / raised against / epitope region							
	Eco2	**E5/G6**	**C16D11**	**2E2**	**B5D9**	**C24B4**	**R31**	**5C2/E10**
	HTNV	HTNV	HTNV	HTNV	HTNV	HTNV	SEOV	ANDV
	1-103	166-175	conf.	n.a.	1-45	conf.	n.a.	40-59
Slovakia	+	+	+	+	-	-	-	-
Slovenia	+	+	+	+	-	-	-	-
aa exchanges in epitope region	S13N T43A V50I S56G	no	n.a.	n.a.	S13N T43A	n.a.	n.a.	T43A V50I S56G

Table 18: Comparison of DOBV isolates SK/Aa and Slo/Af with respect to their neutralisation by convalescent sera from Central European HFRS patients.

serum # [a]	c-FRNT [b]	
	SK/Aa	Slo/Af
1	10,240	640
2	10,240	2,560
3	2,560	640
4	640	160
5	640	160
6	2,560	2,560
7	640	640
8	2,560	10,240
9	640	2,560

[a] Serum samples of HFRS patients clinically characterized elsewhere (sera ##1-6, Sibold *et al*., 2001; serum #7, Klempa *et al*., 2004; sera #8 and #9, our unpublished data). Sera ##1-7 were taken from patients in Germany, #8 and #9 in Slovakia.
[b] Reciprocal end-point titers are given as determined by c-FRNT.

3.5 First genetically confirmed DOBV infection in Central Europe

3.5.1 Clinical description

A 19-year-old male (patient H169) from Ratzeburg (North Germany) was admitted to the local hospital with a five day history of fever (< 39°C), back- and abdominal pains, nausea, vomiting and diarrhoea (two days). Physical examination showed results within normal limits besides occurrence of hepatosplenomegaly. The laboratory tests exhibited very high levels of urea (BUN), serum creatinine and C-reactive protein. Proteinuria and hematuria could be observed (Table 19). He was treated with Paracetamol and with antibiotics (Cotrimoxazol and Ciprofloxacin). With the diagnosis of acute renal failure, after three days (nine days after onset of disease) he was transferred to the University hospital in Lübeck, where he underwent three courses of hemodialysis. Initial complications of hypertension (< 150 mmHg), leucocytopenia and thrombocytopenia were reported. However, the biochemical values gradually returned to normal and the patient was discharged from hospital in good physical conditions five days after third dialysis, about 16 days after onset of disease.

Acute hantavirus infection was identified after the transfer of patient to the University Hospital in Lübeck. By using the POC Hanta test (Erilab Ltd, Finland), for detecting of IgM antibodies to various hantaviruses, IgM antibodies to hantavirus were found in patient's serum taken nine days after onset. The same serum was then examined for the presence of IgM, IgA and IgG antibodies against DOBV and PUUV by in-house ELISA. The titers of IgM, IgA and IgG antibodies to DOBV were determined to be 1:25600, 1:12800 and 1:1200 whereas PUUV-specific antibodies could not be detected. The antibody titer of 1:6400 was detected by in-house immunofluorescence assay (IFA) against DOBV-infected cells (strain Slo/Af). Blood, serum and urine samples taken the same day were also used for detection of hantaviral RNA by RT-PCR.

3.5.2 DOBV infection confirmed by sequence analysis

RNA for RT-PCR was extracted from patient's serum, urine, EDTA-blood and blood stored in AVL Buffer (Qiagene Viral RNA Kit). DOBV S segment specific nested RT-PCR produced a DNA band of expected size (599 nt, nt position 357-955) only for the sample from AVL-blood (Figure 16). Nucleotide sequence of this fragment was determined (557 nt, nt position 378-934 when excluding the primer sequences) and designated DOBV/H169 (H169).

The sequence analysis showed a clear uniqueness of the patient's sequence, sharing

the highest similarity of only 87.4-87.7% nt identity with DOBV-Aa strains from East Slovakia. However, the similarity to DOBV-Af strains was only slightly lower, 86.3-87.0% nt identity. Within the DOBV species, strain Saa/90Aa showed the lowest similarity to the patient sequence, representing only 86.1% nt identity. Although the sequence diversity to other DOBV sequences was very high, most of the nucleotide exchanges represented silent mutations, because percent identity values of the corresponding amino acids (71 aa, aa position 115-185) were very high. Interestingly, the most similar strain appeared to be our new DOBV-Aa isolate SK/Aa (99.4%) (Table 20).

In ML phylogenetic tree based on the 557 nt S segment sequence (nt position 378-934), H169 clustered within the DOBV species and shared a common ancestor with the DOBV-Aa lineage. However, the PUZZLE support for this position of H169 was slightly below the 70% threshold limit (Figure 17). The use of an only 385 nt long fragment (nt position 378-762) allowed us to include the only DOBV-Aa sequence from Slovenia, Slo/9Aa, in the phylogenetic analysis. Interestingly, when we used this shorter alignment including Slo/9Aa, the tree topology remained unchanged but the statistical support, particularly for position of H169, was improved (Figure 18). Two main branches within the DOBV species could be determined. The first well-supported group was formed by DOBV sequences originated from *A. flavicollis* rodents (Slovenia, Slovakia), HFRS patients from Greece and by Saaremaa-Aa strains from Estonia. The second monophyletic group consisted of the DOBV-Aa strains from Slovakia, Slovenia, and Russia and of our new sequence H169. The node giving rise to this group is relatively short (0.01175) although the obtained PUZZLE support of 74% exceeded the 70% threshold limit. H169 represents the most basal strain within this group.

Table 19: Selected laboratory and clinical findings of the H169 patient with acute DOBV infection

Parameter	Patient H169
Sex	male
Age, years	19
Max. serum creatinine (<97 µmol/l)	**1294**
Max. BUN (<49.9 mg %)	**378.6**
Max. extent of albuminuria (<0.3g/d)	**5**
Days of albuminuria	**7**
Erythrocyturia (<4/µl)	**300**
Max. white blood count (<9/nl)	6.4
Min. platelet count (>150 /nl)	**98**
C-reactive protein (<8.2 mg/l)	**42**
Max. systolic blood pressure, mmHg	150
Oedema	no
Days of fever	**5**
Days of oliguria	0
Dialysis treatments (number)	**3**

Normal range values are depicted in brackets, pathological findings are in bold.

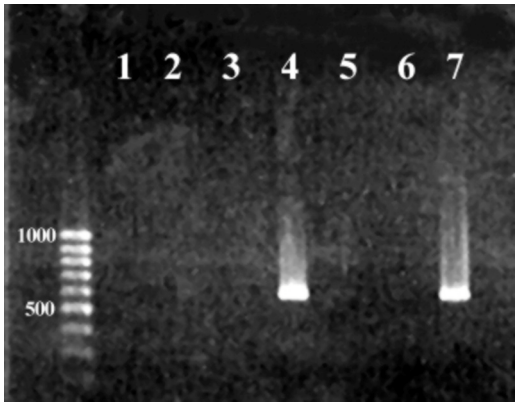

Figure 16: Results of diagnostic DOBV S segment specific PCR with samples from patient H169 visualised by electrophoresis in 1.0% agarose gel after 20 min of staining in ethidium bromide (1 µg/ml) .

Starting material for RNA extraction:
1. serum, 2. EDTA-blood, 3. urine, 4. AVL-blood (560µl AVL Buffer + 140µl blood), 5. Diluted #4 (560µl AVL Buffer + 140µl sample #4), 6. negative control (DEPC water), 7. positive control (DOBV/Slo/Af RNA).

Table 20: Partial S segment nucleotide (599 nt, nt position 357-955) and amino acid (71 aa, aa position 115-185) percent identity of patient-derived sequence H169, other DOBV strains, HTNV, and SEOV

Strain	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.
1. H169	-	87.6	87.4	87.7	87.4	87.2	86.5	86.1	86.7	86.3	86.7	87.0	87.0	86.7	86.5	70.7	70.3
2. Esl/862Aa	98.9	-	93.7	99.1	89.7	89.5	86.5	87.2	84.3	85.6	85.6	85.0	84.7	84.7	84.5	70.7	71.6
3. SK/Aa	99.4	99.4	-	94.2	90.6	90.4	84.9	84.9	84.0	84.7	84.7	84.0	85.0	85.0	84.5	70.7	71.0
4. Esl/194Aa	98.9	100	99.4	-	89.9	89.7	86.7	87.0	85.2	85.8	86.1	85.2	84.9	84.9	85.0	70.9	71.6
5. Kur/53Aa	98.3	98.3	98.9	98.3	-	99.8	87.4	87.0	87.2	87.2	87.2	87.2	87.7	87.7	87.0	71.9	70.9
6. Kur/44Aa	97.8	97.8	98.3	97.8	99.4	-	87.2	86.8	87.0	87.0	87.0	87.0	87.6	87.6	86.8	71.8	70.9
7. Saa/160v	96.7	96.7	97.2	96.7	96.2	95.6	-	98.2	87.4	87.9	87.6	86.8	86.8	86.8	86.3	70.7	71.2
8. Saa/90Aa	96.7	96.7	97.2	96.7	96.2	95.6	100	-	86.8	87.9	87.6	86.5	86.5	86.5	85.9	70.9	70.7
9. Esl/400Af	98.3	98.3	98.9	98.3	97.8	97.2	97.2	97.2	-	96.2	95.8	96.4	94.7	94.7	96.4	70.0	69.1
10. Slo/Af	97.2	97.8	97.8	97.8	96.7	96.2	97.2	97.2	98.9	-	99.2	96.0	95.3	95.3	96.2	70.5	69.4
11. DOB-Af1	97.2	97.8	97.8	97.8	96.7	96.2	97.2	97.2	98.9	100	-	96.4	94.9	94.9	96.2	70.1	69.6
12. DOB-PR	98.3	98.3	98.9	98.3	97.8	97.2	97.2	97.2	100	98.9	98.9	-	95.6	95.3	98.7	70.1	70.0
13. DOB-EA	98.3	98.3	98.9	98.3	97.8	97.2	97.2	97.2	100	98.9	98.9	100	-	99.6	95.8	71.0	69.8
14. DOB-SZ	98.3	98.3	98.9	98.3	97.8	97.2	97.2	97.2	100	98.9	98.9	100	100	-	95.5	71.2	69.8
15. DOB-TD	98.3	98.3	98.9	98.3	97.8	97.2	97.2	97.2	100	98.9	98.9	100	100	100	-	70.7	69.8
16. SEOV	75.6	75.6	76.2	75.6	76.2	75.6	75.1	75.1	76.2	75.1	75.1	76.2	76.2	76.2	76.2	-	68.7
17. HTNV	77.2	76.7	77.2	76.7	77.2	77.2	77.2	77.2	77.8	76.7	76.7	77.8	77.8	77.8	77.8	76.2	-

The identity values were calculated using Clustal method. The percentage identities for nucleotide (above the diagonal) and amino acid (below the diagonal) sequences are presented.

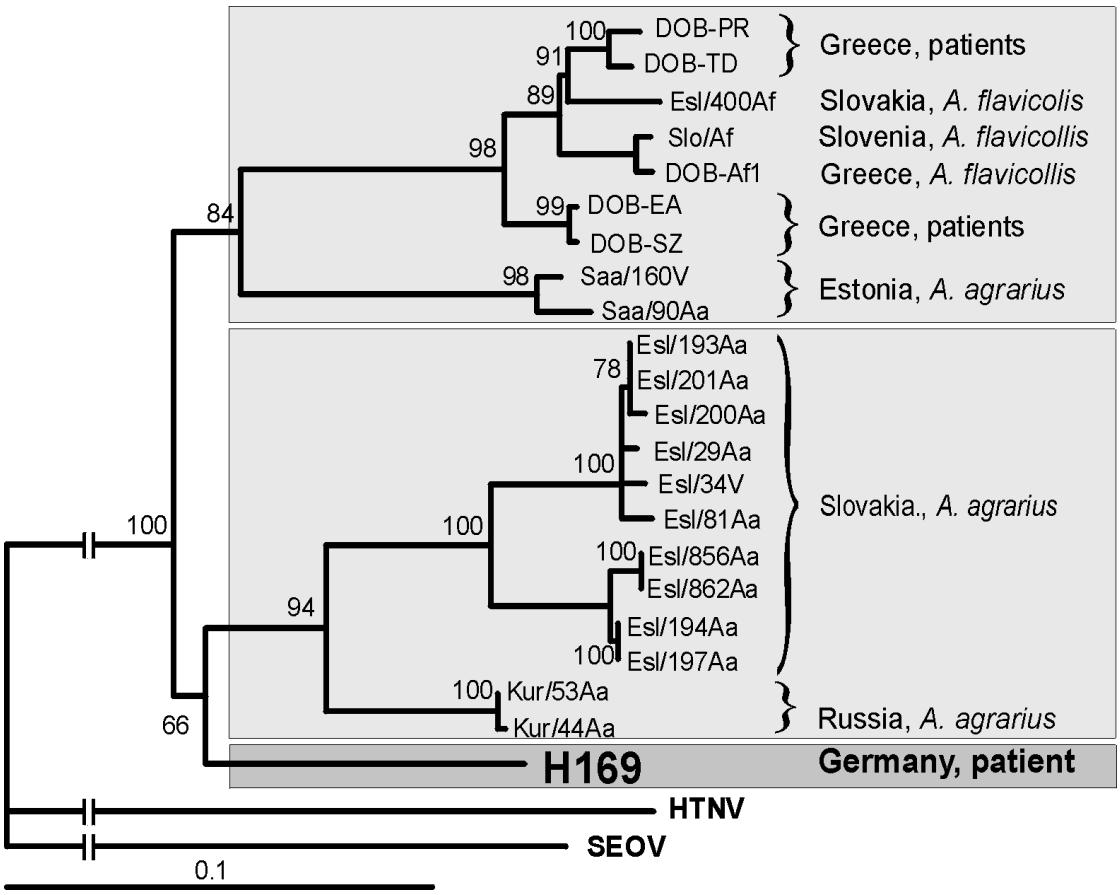

Figure 17: Phylogenetic ML tree based on the partial S segment nucleotide sequences (378-934 nt) of H196 and other DOBV strains, computed with TREE-PUZZLE.

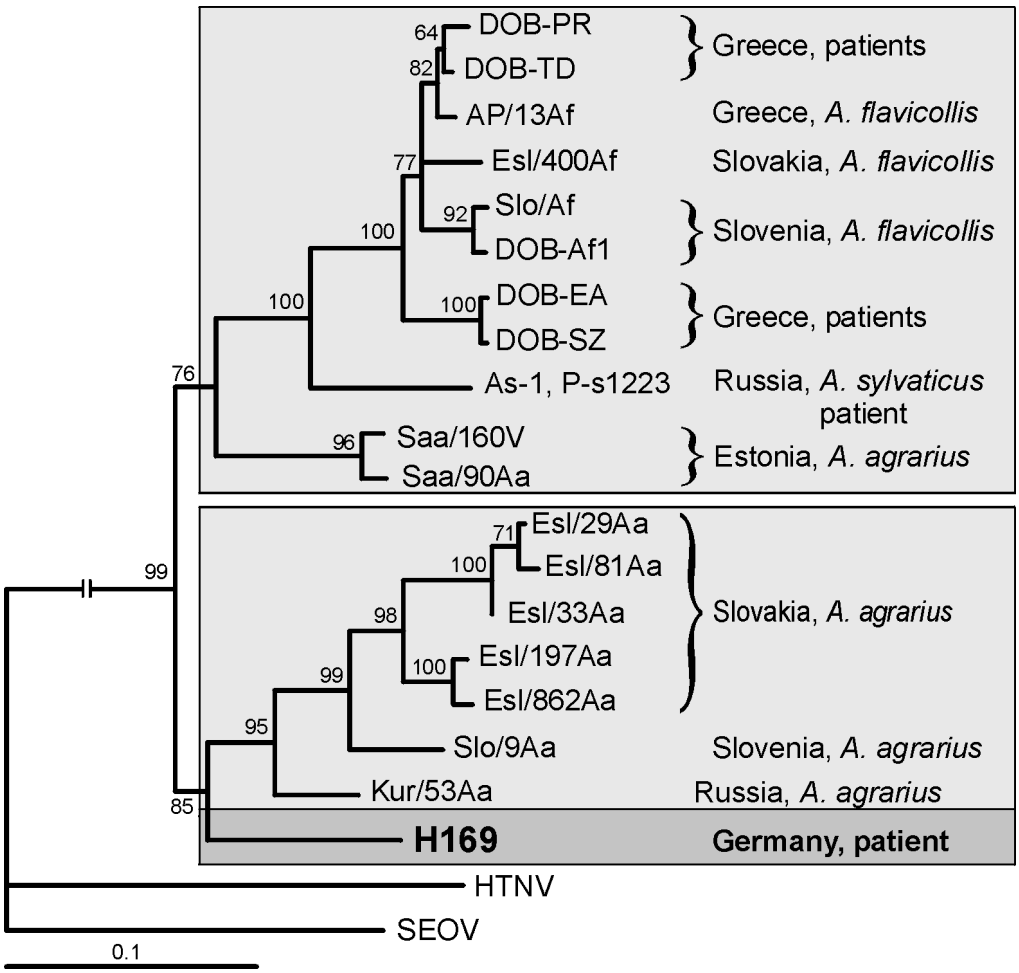

Figure 18: Phylogenetic ML tree based on the partial S segment nucleotide sequences (378-762 nt) of H196 and other DOBV strains, computed with TREE-PUZZLE.

3.6 First report of DOBV-Af associated HFRS cases in Slovakia

Most of the HFRS cases due to DOBV infection in Slovakia are occurring in the Eastern part of the country where *A. agrarius* is dominating over *A. flavicollis* in rodent population. Several DOBV-Aa strains and only one DOBV-Af strain were also directly detected in local *Apodemus* populations. Therefore, it was interesting to investigate two DOBV-associated HFRS cases from West Slovakia, where *A. flavicollis* but not *A. agrarius* is present in nature.

A 13-year-old boy (patient A) from Trencin (West Slovakia) had a three-day history of fever (<39.0 °C), abdominal pain and vomiting. He was treated as an outpatient with Amoxycillin (1g tid po) and Bactisubtil® (dried *Bacillus cereus* spores, 105 mg/d po). Because of persisting abdominal pains and nausea, diarrhoea and urticaria on skin, he was admitted to the hospital eight days after the onset of symptoms. The laboratory tests (Tables 21 and 22) exhibited very high levels of urea (>40mmol/L), serum creatinine (>900 µmol/L), and C-reactive protein (34.5 mg/L). With the diagnosis of acute renal failure, he was transferred to intensive care unit where he underwent two courses of hemodialysis (at days 9 and 10 after onset). After dialysis the biochemical values gradually returned to normal and patient recovered within five days. He was discharged with normal laboratory and clinical findings 14 days after hospitalisation, 22 days after onset of illness.

A 11-year-old boy (patient B), the brother of patient A, was admitted to the hospital one day later than his brother. Four days before admittance, the patient suffered from fever (38.7°C), nausea, vomiting, abdominal pain and petechiae on face and neck. Laboratory tests (Tables 21 and 22) revealed high levels of urea (22.6 mmol/L, serum creatinine (290 µmol/L) and 10-fold elevation of liver enzymes. Because of similar symptoms of his brother, he was transferred to intensive care unit with a diagnosis of a hepatorenal syndrome at the same day as his brother. However, dialysis was not necessary. Under parenteral rehydratation treatment, his clinical and laboratory parameters gradually improved to normal values. During the hospitalisation, he developed bronchopneumonia which was treated with parenteral antibiotics. He was discharged from hospital in good physical condition 21 days after onset of disease.

With routine diagnostic methods, no evidence for acute bacterial, fungal, or viral infections was found. When patients were discharged from hospital, the etiology of disease was still unknown. Patients reported that they had been regularly performing water sports on a natural water canal near Trencin. After intervention of the local epidemiologist (Dr. Stankovicova, Trencin), the convalescent sera were sent to our collaboration partner, Institute of Virology, Bratislava, Slovakia, as late as 17 months after the disease. IgG

antibodies in sera of both patients were tested by nucleocapsid-protein specific ELISA using PUUV and HTNV antigens (Progen GmbH) and found to be positive. By in-house immunofluorescence assay (IFA), antibody titers were found to be higher against DOBV and HTNV than against PUUV suggesting DOBV infection (Table 23).

This virological diagnosis was then confirmed by FRNT. We have determined the neutralising activities of sera #1 against the following viruses; DOBV, HTNV, SEOV, and PUUV. An at least fourfold higher reciprocal titer was found against DOBV strain Slo/Af when compared with the other viruses (Table 23). However, DOBV strain originating from *A. agrarius* was not available for us at that time. After the isolation of a DOBV-Aa strain from Slovakia the comparison with DOBV-Aa could be done. Sera obtained 43 months after onset of disease were analysed and showed fourfold higher reciprocal titer against Slo/Af than against SK/Aa (Table 23) indicating that DOBV-Af was responsible for the infection of both patients.

Table 21: Clinical and laboratory data of two DOBV-Af patients according to criteria for estimating severity of the various phases of HFRS

Phase	Observation	Criteria for severity*			Patient	
		Mild +	Moderate ++	Severe +++	A	B
Febrile	Max. temperature	<39	39-40	>40	+	+
	Days temp. over 38 °C	1-5	5-7	>7	+	+
	Petechiae, flushing	0/+	++/+++	++++	+	+
	Max WBC count	<14.500	14.5-30x10^3	>30.000	+	++
Hypotensive	Max. haematocrit (%)	<50	51-56	>57	+	+
	Min. systolic BP (mmHg)	96-120	81-95	<80	+	+
	Hours of hypotension	<24	24-48	>48	+	+
	Min. platelets	>90.000	4-9x10^4	<40.000	+	+
Oliguric	Min. haematocrit	>45	35-44	<35	++	++
	Max. systolic BP (mmHg)	<140	141-170	>170	++	+
	Days of hypertension	<1	1-2	>2	++	+
	Max. BUN (mg %)	20-79	80-149	>150	+++	++
	Days of proteinuria	<4	4-5	>5	+++	+
Diuretic	Max. daily urine vol.	<3.4l	3.5-4.9l	>5l	+	+

Criteria for estimating severity of the various phases of HFRS according to (Lee and van der Groen, 1989).

Table 22: Further laboratory and clinical data of the Trencin patients of acute DOBV infection

Parameter	Patient	
	A	B
Sex	male	male
Age, years	13	11
Max. serum creatinine (<102µmol/l)	**930**	**304**
Serum creatinine at discharge	85	64
Min. platelet count (>140 Gpt/l)	189	**137**
Max. platelet count (<440 Gpt/l)	377	366
Max. white blood count (<10.8 Gpt/l)	9.4	**16.9**
C-reactive protein (<8.2 mg/l)	**34.5**	**27.37**
GOT (<0.49 µmol/l/s)	**0.76**	**2.63**
GPT (<0.57 µmol/l/s)	**1**	**2.33**
Max. extent of proteinuria (<0.15g/d)	**0.256**	**0.258**
Max. systolic blood pressure, mmHg	150	130
Systolic blood pressure at discharge, mmHg	110	115
Oedema	no	no
Days of fever	**3**	**3**
Days of oliguria	0	0
Dialysis treatments (number)	**2**	0

Normal range values are depicted in brackets, pathological findings are in bold.

Table 23: Detection of hantavirus-specific antibodies in serum samples of HFRS patients from West Slovakia

Serum[a]	ELISA [b]			IFA [c]			c-FRNT [d]					
	HTNV	DOBV	PUUV	HTNV	DOBV	PUUV	DOBV-Af	DOBV-Aa	HTNV	SEOV	PUUV	TULV
Patient A												
#1	pos.	n.d.	pos.	6400	6400	400	2560	n.d.	40	640	40	40
#2	pos.	pos.	pos.	n.d.	25600	n.d.	**10240**	**2560**	n.d.	n.d.	n.d.	n.d.
Patient B												
#1	pos.	n.d.	neg.	6400	6400	200	2560	n.d.	160	160	40	40
#2	pos.	pos.	pos.	n.d.	12800	n.d.	**2560**	**640**	n.d.	n.d.	n.d.	n.d.

[a] Sera were taken 17 months (#1) and 43 months (#2) after onset of disease
[b] s/c ratios > 1.5 were taken to be positive. The IgG screening ELISA (Progen GmbH, Heidelberg, Germany) was used.
[c] Reciprocal end-point titers are given. Vero-E6 cells used in IFA were infected by HTNV (strain 76-118), DOBV (strain Slovenia) or PUUV (strain Sotkamo) .
[d] Reciprocal end-point titers are given. The chemiluminescence focus reduction neutralisation test was undertaken by use of DOBV-Af (strain Slovenia), DOBV-Aa (strain SK/Aa), HTNV (strain 76-118), SEOV (strain 80-39), PUUV (strain Sotkamo), and TULV (strain Moravia).

3.7 Occurrence of renal and pulmonary syndrome in a region of North-East Germany where Tula hantavirus circulates

3.7.1 HFRS clinical case associated with TULV infection

3.7.1.1 Clinical description

A male 43-year-old patient from a rural region near Cottbus (Northern German Plain) was admitted to the hospital. Since the previous day he had suffered from fever (40.0 ^{0}C), chills, headache and left-thoracic, breathing-dependent pain. The X-ray examination showed an infiltration within the left pulmonary midzone. The laboratory tests exhibited elevated levels of serum creatinine (116 μmol/L ≅ 1.3 mg/dL), C-reactive protein (91.55 mg/L) and a deviation to the left in the differential blood count. Platelet count, serum bilirubin and transaminase values were found to be normal. However, proteinuria, erythrocyturia, urobilinogenuria and bilirubinuria have been detected in urine. During clinical course, the proteinuria reached a value of 4.55 g/d. No oliguria was observed but a moderate polyuria did occur at day 7 and the following days. With routine diagnostic methods, no evidence for acute bacterial, fungal, or viral infections (with the exception of elevated hantavirus antibody titers, see below) was found to explain the nephritis and pneumonia. A spontaneous remission of clinical symptoms and laboratory values was observed during supportive treatment of the patient. The man had not visited other countries during the previous years. However, he reported that he had frequently seen and trapped rodents in his rural living place.

At day 15 the patient was discharged from the clinic, however, at day 21 he was hospitalized again with symptoms of fever (up to 40 ^{0}C), unproductive cough and pain in the left thorax. Diagnostic radiology now demonstrated an infiltration in the basolateral segment of left lower lobe, whereas the primary infiltration in the left pulmonary midzone was no longer detectable. Again, elevated levels of serum creatinine and C-reactive protein were found, but this time no proteinuria occurred. Ribavirin treatment (Rebetol^{R} 1g/d) was started and maintained over two weeks. Within one week after the second admission (day 28 after onset of illness) the patient was free of fever and biochemical values gradually returned to normal. At day 36 after onset the patient was finally discharged from the clinic. In the follow-up period no clinical or laboratory deviations were observed. In particular, there was a complete remission of the pulmonary infiltration.

3.7.1.2 Serological data

The hantavirus antibody titers against PUUV antigen as determined by IFA (Progen GmbH, Heidelberg, Germany) increased from 1:128 (day 2 after onset), through 1:256 (day 12) to 1:3,200 (day 38) demonstrating an acute hantavirus infection (laboratory Dr. Limbach & Partners, Heidelberg, Germany). The first serum (#1) studied by us was taken 27 days after onset of symptoms. The patient gave his informed consent to these investigations. IgG antibodies were tested by nucleocapsid-protein specific ELISA using PUUV and HTNV antigens (Progen GmbH) and found to be positive. IgM antibodies could not be detected by IgM specific ELISA (Progen GmbH). By in-house immunofluorescence assay (IFA), antibody titers were detected against PUUV- (1:512), TULV- (1:512), HTNV- (1:32) and DOBV-infected cells (1:256). A second serum sample (#2) taken 7 months later confirmed these findings (Table 24). The slightly higher IFA antibody titers against the viruses of the PUUV/TULV group as compared with DOBV/HTNV could indicate an infection by PUUV or a related hantavirus.

The chemilumuniscent focus reduction neutralisation test (c-FRNT), which is performed under biocontainment level-3 conditions, is the only method for fine-typing of sera positive for hantavirus antibodies. By c-FRNT we have determined the neutralising activities of sera #1 and #2 against the following viruses; PUUV, TULV, HTNV, DOBV, and SEOV. As shown in Table 24, an at least fourfold higher reciprocal titer was found against TULV when compared with the other viruses. This supports the assumption that TULV was the virus responsible for the infection of the patient. Nevertheless, it cold not be completely excluded that the infection was caused by a TULV-related, albeit unidentified virus not present in the FRNT virus collection. It was thus important to show that TULV is endemic in this region of Germany.

3.7.2 Detection of TULV in *Microtus arvalis* from North-East Germany

In the course of our epidemiological studies on hantavirus distribution in Germany, rodents were trapped and screened for hantavirus infection. Infected *M. arvalis* rodents could be captured in North-East (NE) Germany at two sites a few kilometers far from the home village of the patient. From tissues of three out of 18 tested animals hantaviral RNA could be detected by RT-PCR and the nucleotide sequence of the complete S segment was determined.

In all three cases (samples no. D5, D17, D63) the S segments were found to be 1,852 nt in length (1,828 nt, when excluding the primer sequences) and revealed an ORF1 encoding a nucleocapsid protein of 430 aa, flanked by a 5‘ NCR of 42 nt and a 3‘ NCR of 517 nt. In addition to the ORF1, all three samples contained the potential second ORF2, which overlaps the nucleocapsid protein ORF1 and is 273 nt long. The ORF2 reading frame is in +1

position with respect to the ORF1, encoding a putative nonstructural protein of 90 aa.

Percent sequence identities on the nucleotide and amino acid level for the three samples in comparison to all other European Tula viruses are given in Table 25. Sequence analysis revealed that their genetic difference followed the geographic distance of the trapping sites: Tula D5 and Tula D17 (trapping sites < 1km apart) showed 99.8% (nt) and 99.7% (aa) identities, whereas Tula D63 (trapping site about 3 km apart) showed identities of 99.4-99.6% (nt) and 99.3-99.5% (aa), respectively. The most similar sequences were found in the Polish strains (91.2-91.5% nt and 98.6-90.0% aa identity). Similarity to all other strains was a approximately on the same level (83.6-84.6 nt, 96.5-97.9 aa identity).

A summary of the amino acid changes between the Tula strains is shown in Table 26. At five positions, unique amino acid changes consistent among the NE German and Polish samples were found that distinguished them from the other Tula isolates. At position 263, aa Tyr could be found only in two of our strains. All other TULV strains show a non-conservative exchange to aa Asn at that position.

Comparison of the 3‘ NCR of the NE German strains revealed > 99.6% nt identity. Comparison with other Tula strains yielded nt identities of about 80.6%-90.9%. Unique for our samples a consistent two nt insertion was observed immediately downstream of the stop codon (nt 1340-1341), as well as another at nt 1447-1448. The 16 nt long deletion that was shown to be typical for Tula strains from Southern Central Europe (Sibold *et al*., 1999a) was neither found in the NE German nor the Polish strains.

Including known complete S segment sequences of other TULV strains, a ML phylogenetic tree was constructed (Figure 19). The results show that the NE German strains form a monophyletic group with the Polish strains. TULV strains can be divided into three distinct, well-supported lineages. The first lineage is represented by strains from Russia (East Europe), the second by strains from the Czech Republic and West Slovakia, Croatia and South Germany (Southern Central Europe) and the third by strains from North-East Germany and Poland (North-Central Europe). Regarding the third genetic lineage it seems to be reasonable to conclude that its distribution follows the Northern German Plain which extents to Poland in the East.

Table 24: Detection of hantavirus-specific antibodies in serum samples of the patient

Serum [a]	ELISA [b]		IFA [c]				c-FRNT [d]				
	PUUV	HTNV	PUUV	TULV	HTNV	DOBV	PUUV	TULV	HTNV	DOBV	SEOV
#1 (H145)	pos.	pos.	512	512	32	256	< 40	160	< 40	< 40	< 40
#2 (H53)	pos.	pos.	1024	256	64	256	40	160	40	< 40	< 40

[a] Sera were taken 4 weeks (#1) and 7 months (#2) after onset of disease
[b] s/c ratios > 1.5 were taken to be positive. IgG screening ELISAs (Progen GmbH, Heidelberg, Germany) were used.
[c] Reciprocal end-point titers are given. Vero-E6 cells were infected by PUUV (strain Sotkamo), TULV (strain Moravia), HTNV (strain 76-118) or DOBV (strain Slovenia) and used in immunofluorescence assay (IFA).
[d] Reciprocal end-point titers are given. The chemiluminescence focus reduction neutralization test was undertaken by use of PUUV (strain Sotkamo), TULV (strain Moravia), HTNV (strain 76-118), DOBV (strain Slo/Af), and SEOV (strain 80-39).

Table 25: Percent sequence identities among North-East German and other European TULV strains based on entire S segment and N protein sequences

Strains *	D5		D17		D63	
	nt	aa	nt	aa	nt	aa
D5	-	-	99.8	99.7	99.4	99.3
D17	99.8	99.7	-	-	99.6	99.5
D63	99.4	99.3	99.6	99.5	-	-
Poland	91.5-91.6	98.8-99.0	91.4-91.5	98.6-98.8	91.2-91.3	98.6-98.8
Russia	84.4-85.6	96.9-97.4	84.4-85.5	96.5-97.2	84.3-85.4	96.5-97.2
East Slovakia	84.3-84.8	97.6-97.9	84.2-84.7	97.4-97.6	84.1-84.6	97.4-97.6
West Slovakia	84.5	97.4	84.2-84.3	97.2	84.2-84.3	97.2
Czech Republic	83.7-84.6	96.7-97.4	83.6-84.3	96.5-96.9	83.6-84.3	96.5-96.9
Croatia	84.6	97.2	84.6	96.9	84.5	96.9
South Germany	84.2	96.2	84.2	96.0	84.2	96.0

* The European TULV strains used in this analysis were grouped according to their geographical of origin. Acc. numbers of the strains included in these groups are listed in Table 26.

Table 26: Amino acid exchanges in N protein of TULV strains

	polymorphic sites																							
Acc. No.	2	3	42	48	60	84	121	127	134	165	234	250	252	254	256	258	263	268	305	312	323	387	404	country of origin
Z30941	S	Q	N	R	E	I	A	I	V	F	E	Q	S	G	E	E	N	I	A	A	I	M	D	
Z30942	S	Q	N	R	E	L	A	I	V	F	E	Q	S	G	E	E	N	I	A	A	I	M	D	
Z30943	S	Q	N	R	E	L	A	I	V	F	E	Q	S	G	E	E	N	I	A	A	I	M	D	Russia
Z30944	S	Q	N	R	E	I	A	I	V	F	E	Q	S	G	E	E	N	I	A	A	I	M	D	
Z30945	S	Q	N	R	E	L	A	I	V	F	E	Q	A	G	E	E	N	I	A	A	I	M	D	
Y13979	S	Q	S	R	D	L	S	I	V	F	E	Q	A	G	E	D	N	L	A	A	I	T	D	E Slovakia
Y13980	S	Q	S	R	D	L	S	I	V	F	E	Q	A	G	E	D	N	L	A	A	I	T	D	
Z48235	S	Q	S	R	D	L	S	T	I	Y	E	S	-	S	E	D	N	L	A	A	I	T	D	W Slovakia
Z68191	S	Q	S	R	D	L	S	T	I	Y	E	S	-	S	E	D	N	L	A	A	I	T	D	
Z48573	S	Q	S	G	D	L	S	T	I	Y	E	S	-	S	E	D	N	L	A	R	I	T	S	
Z48574	S	Q	S	G	D	L	S	T	I	Y	E	S	-	S	E	D	N	L	A	R	I	T	S	
Z48741	S	Q	S	R	D	L	S	T	I	Y	E	S	-	S	E	D	N	L	A	R	I	T	D	
Z49915	S	Q	S	R	D	L	S	T	I	Y	E	S	-	S	E	D	N	L	A	R	I	T	D	Czech Rep.
Z69991	S	Q	S	R	D	L	S	T	I	Y	E	S	-	S	E	D	N	L	A	R	I	T	D	
AJ223601	S	Q	S	R	D	L	S	T	I	Y	E	S	-	S	E	D	N	L	A	R	I	T	D	
AJ223600	S	Q	S	R	D	L	S	T	I	Y	E	S	-	S	E	D	N	L	A	R	I	T	S	
AF164094	S	Q	S	R	D	L	S	T	I	Y	E	N	-	S	E	D	N	L	A	A	I	T	D	Croatia
AF164093	S	Q	S	Q	D	L	S	M	I	Y	E	S	-	S	E	D	N	L	A	A	I	T	D	S Germany
D5	S	Q	S	R	D	L	S	I	V	Y	D	Q	G	N	D	E	Y	L	A	A	V	M	D	
D17	S	Q	S	R	D	L	S	I	V	Y	D	Q	G	N	D	E	Y	L	G	A	V	M	D	NE Germany
D-63	S	Q	S	R	D	L	S	I	V	Y	D	Q	G	N	D	E	N	L	G	A	V	M	D	
Af063892	T	E	S	R	D	L	S	M	V	Y	D	Q	G	N	D	E	N	L	A	A	V	M	D	Poland
Af063897	T	E	S	W	D	L	S	M	V	Y	D	Q	G	N	D	E	N	L	A	A	V	M	D	

Only informative sites are shown. Amino acid residues unique for NE German and/or Polish strains are grey boxed. TULV lineages as defined in Figure 19 and recombinant strains from East Slovakia are separated by empty lines.

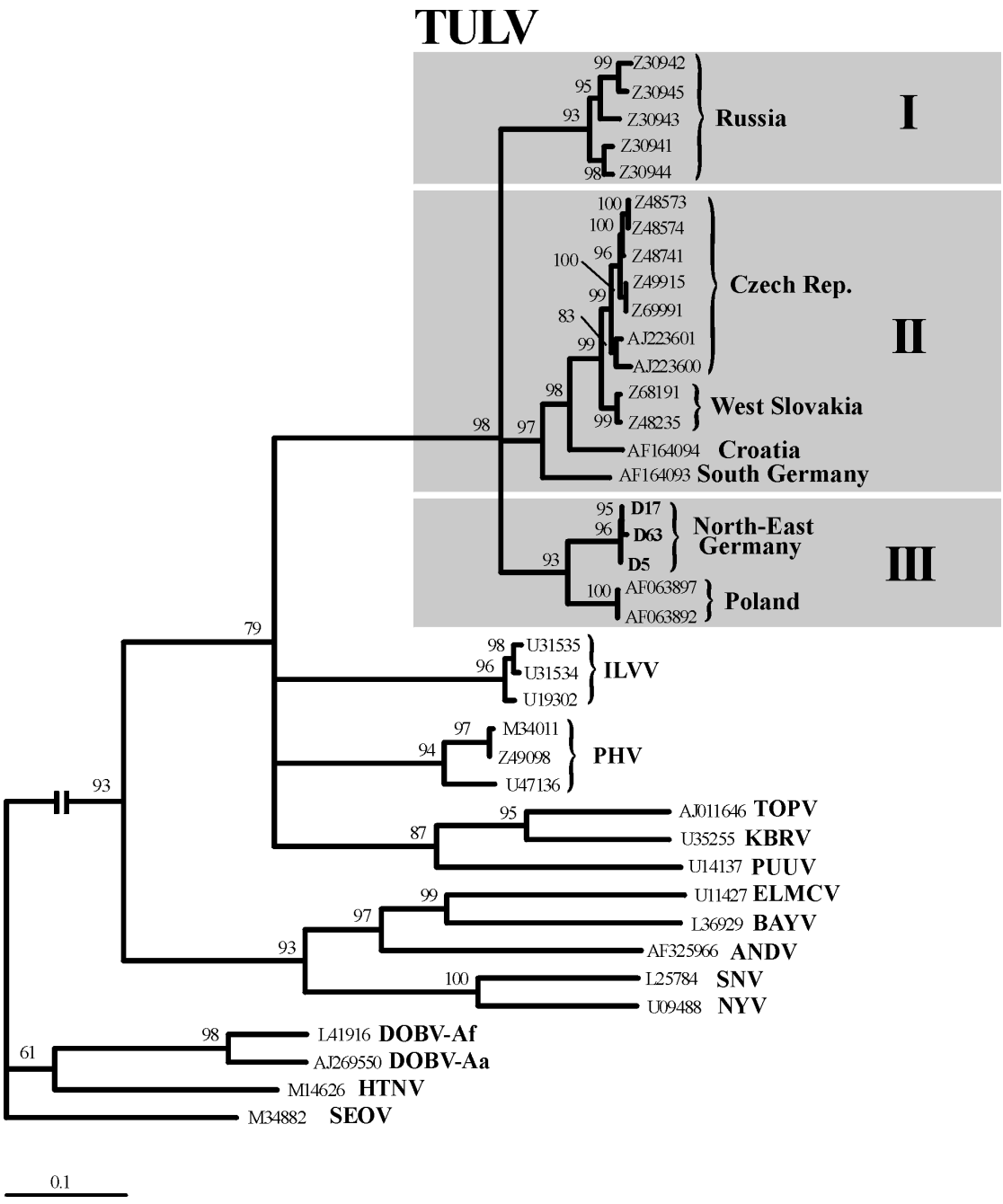

Figure 19: ML phylogenetic tree of TULV strains based on complete S segment nucleotide sequences

TULV lineages are grey boxed. North-East German strains are in bold.

4. DISCUSSION

4.1 Phylogenetic classification of Central European DOBV-Aa and DOBV-Af strains

Our sequence comparisons and phylogenetic analyses showed that the Central European DOBV strains cluster within two separate clades according to their natural host (DOBV-Aa vs. DOBV-Af). In the S segment these strains are related to the *A. agrarius*-derived DOBV strains from Russia (Kurkino-Aa) but quite less to the *A. flavicollis*-derived virus strains (Slo/Af, Esl/400Af, AP/Af) and the Saaremaa-Aa strains.

In the M segment, the analysed Central European DOBV strains (Esl/862Aa, SK/Aa) were found to be more related to the strain Saa/160V than to the *A. flavicollis* DOBV strains. This demonstrates a strong phylogenetic relationship between the *A. agrarius* DOBV strains (DOBV-Aa) on the one hand and the *A. flavicollis* DOBV strains (DOBV-Af) on the other. However, both DOBV lineages could be clearly distinguished from the most closely related virus species, i.e., HTNV or SEOV.

4.2 Genetic reassortment of genome segments in Saa/160V

Our phylogenetic analyses of DOBV complete S and M segment sequences showed that Saa/160V strain possess a DOBV-Af like S segment, whereas its M segment groups together with the Central European strain Esl/862Aa as well as with the new DOBV-Aa isolate SK/Aa. These results gave evidence that Saa/160V was involved in one or more reassortment processes. Identification of the partial L segment sequence of the new isolate SK/Aa allowed additional insights into the interesting topic of reassortment during DOBV evolution. The ML analysis showed that not only in S but also in L segment, the Saa/160V strain (although found in *A. agrarius* rodents) is more related to DOBV-Af strains and only its M segment resembles that of DOBV-Aa strains (Figure 20). This suggests that the M segment encoding the viral glycoproteins is crucial for the hantavirus host specificity. Although at the current stage of knowledge other scenarios cannot be ruled out, it might be concluded that Saa/160V was originally an *A. flavicollis*-associated virus which has switched to *A. agrarius* during its evolution. To overcome the virus-host specificity barrier and to establish a persistent infection in the new host, it could have acquired an *A. agrarius*-specific

M segment from the DOBV-Aa lineage. Further studies on biological consequences of reassortment in hantaviruses are needed. The availability of “non-reassorted” DOBV-Af (Slo/Af) and DOBV-Aa (SK/Aa) isolates as well as the apparently reassorted Saa/160V will now allow to study the importance of this process in vitro.

Figure 20: Schematic illustration of the proposed scenario of genetic reassortment during DOBV evolution. S and L segments of Saaremaa virus are phylogenetically related to their counterparts in DOBV-Af, whereas the M segment (determining the antigenicity of the virus envelope) resembles that of DOBV-Aa.

In its cross-neutralisation behaviour, Saa/160V has been found to differ from Slo/Af (Brus Sjolander *et al*., 2002). This can be explained by the fact that there are 72 aa differences between the glycoproteins of the two virus isolates, Saa/160V and Slo/Af. Only 38 of them are potentially host-specific (see below). It seems to be reasonable to conclude that Saa/160V is not a good virus prototype of the Central European DOBV-Aa strains because of its DOBV-Af-like S and L segments and the large number of aa exchanges in the M-segment encoded glycoproteins which are not typical for the Central European DOBV-Aa strains.

There is one other example of natural genetic reassortment in hantavirus evolution; reassortment of genomic RNA segments has been also found between SNV strains (Henderson *et al*., 1995; Li *et al*., 1995). Moreover, genetic reassortants involving S and M segments were detected after mixed infections in tissue culture by using two distinct strains of SNV. One virus reassortant was observed also after mixed infections between SNV and genetically more distant Black Creek Canal virus (Rodriguez *et al*., 1998). This together with our own results indicates that genetic reassortment is not an uncommon process in

hantavirus evolution.

For viruses with segmented genomes, reassortment is an additional way of generating variation and antigenic alteration. A prominent example is the influenza virus. The most abrupt changes in antigenic specificity occur through hemagglutinin and neuraminidase genes reassortment. The mixing of segments from avian and mammalian influenza viruses has in certain cases yielded a very high fitness, as exemplified by the Asian (1957) and Hong Kong (1968) pandemic flu viruses (see Hungnes *et al*., 2000; Hilleman, 2002). It has to be evaluated whether the genetic reassortment in hantaviruses might have similar consequences concerning altered host specificity or pathogenicity towards humans.

4.3 Genetic recombination between DOBV-Af and DOBV-Aa lineages

Natural co-infection of the same host animal and the same host cell with different virus strains is the precondition also for the occurrence of genetically recombined viruses during their evolution. It was recently shown that recombination between different members of the hantavirus species Tula had occurred in their evolution; this was the first example of natural homologous recombination in negative-stranded RNA viruses (Sibold *et al*., 1999a).

Now, we have found two parts in the M segment of strain Esl/862Aa and one in the S segment of Esl/8xxAa that show signs for an evolutionary history differing from the rest of the sequence. While two of the areas (S 400-600 nt and M 1972-2211 nt) show only moderate puzzle support values between 62% and 64% in the phylogenetic trees, the area from 810 – 1059 nt of M segment with a support of 81% gives some evidence for an event changing the evolutionary history in respect to the flanking sequences.

Two events could explain the creation of this significant nucleotide sequence exchange at M segment position 810 - 1059. First, directed selection could have led to such differences in parts of the sequence, but an area of 250 nt should be most likely too long to be generated by directed selection. The other possible scenario consists in the occurrence of genetic recombination. As usually in case of recombination, one would expect one bootscanning curve dropping from high to very low values, while another curve comes up, indicating an exchange of genetic material leading to a recombinant query sequence from parental sequences related to the ones belonging to the changing curves. The pattern found here (Figure 10), shows that in the region between nt positions 810 - 1059 the alignment of the query sequence (Esl/862Aa) with Saaremaa-Aa is interrupted and no curve comes up reaching the threshold value. Two recombination scenarios are most likely: First,

recombination between a Saaremaa-like ancestor of Esl/862Aa with a member of a (so far) unknown DOBV lineage older than the sequences we used, creating the recombinant virus Esl/862Aa. Or, secondly, an Esl/862Aa-like ancestor of Saaremaa has recombined with an DOBV-Af like lineage forming a recombinant Saaremaa virus.

However, the phylogenetic analysis of this recombination event might be influenced by the short extent (249 bp) of the region. Hence, this issue needs to be further evaluated, taking into account biological properties of M segment gene products which are currently not very well characterised.

The availability of more sequenced virus strains would be helpful to further elucidate the scenario of recombination processes in the evolution and intraspecies diversity of Dobrava virus. Nevertheless, the recombination event in DOBV evolution, after similar findings for TULV in its natural evolution (Sibold *et al*., 1999a) and under *in vitro* conditions (Plyusnin *et al*., 2002), offers another example of natural homologous recombination in the evolution of hantaviruses. In addition, there are indications for recombination events within the PUUV species, too (Sironen *et al*., 2001).

4.4 Role of genetic differences for host adaptation

The sympatric occurrence of DOBV strains in two different host species, *A. agrarius* and *A. flavicollis*, opens the chance of a comparative genetic analysis which could contribute to an understanding of adaptation to different hosts leading to genetic differences between the virus lineages. This might allow conclusions also for suggested pathogenicity differences towards humans.

Altogether, three aa differences between DOBV-Aa and DOBV-Af strains in N protein and 38 aa exchanges in G1/G2 have been identified. This list of candidate host-specific determinants can be probably reduced when more complete sequences will be elucidated and included in the analysis.

However, there are certain limits in possible conclusions from aa comparisons even in the case that an enhanced number of DOBV-Aa and DOBV-Af sequences will be available in the future: (i) Secondary (”suppressor”) aa exchanges could restore the host-dependent functionality of a protein and could mask true aa differences, (ii) the role of the nucleotide sequence independent of their coding properties for host-adaptation of a virus (nucleic acid itself as subject of genetic selection) is unknown yet. Probably important are also the sequences of NCR regions because of their role in replication and transcription regulation (Jonsson and Schmaljohn, 2001) and binding of N protein (Gott *et al*., 1993). Lundkvist *et al*.

(1997b) have shown that single mutations in the NCR of the PUUV (strain Kazan) S segment could be associated with adaptation of this virus to bank vole *versus* cell culture. However, in our case it is difficult to define the importance of particular nucleotide exchanges in the NCRs because of the low degree of homology between the DOBV strains in these genomic regions (especially in the M segment 3'NCR).

An ultimate proof of the role of distinct amino acid exchanges in the DOBV proteins for host adaptation (*A. flavicollis versus A. agrarius*) would only be possible by using reverse genetics systems to generate infectious clones. The development of this field of research is still in its first steps for bunyaviruses in general and hantaviruses in particular (Jonsson and Schmaljohn, 2001; Flick *et al*., 2003). Moreover, one would need sophisticated *Apodemus* animal models that allow cross-infection experiments in the laboratory. Moreover, a "read-out" systems to compare the antiviral responses of endothelial cells, as recently used for HTNV and TULV (Kraus *et al*., 2004), trigered by various reverse genetics-generated DOBV clones would allow to study the role of these amino acid exchanges for the pathogenicity differences towards humans.

There are some indications that viruses of the DOBV-Aa and the DOBV-Af lineages could not only differ in their host reservoir but also in their pathogenicity towards humans (Sibold *et al*., 2001). So, the detailed comparison of these virus lineages could also improve the knowledge on pathogenicity-specifying genetic determinants. Given the present stage of knowledge we may be correct to state that the S segment should be less relevant for the different pathogenicity; despite possessing an S segment related to the highly virulent DOBV-Af, Saa/160V is presumed to be less pathogenic as it seems to be the case for the Central and East European DOBV-Aa strains (Plyusnin and Morzunov, 2001). It seems reasonable that the glycoproteins are the main pathogenicity determinants since they are clearly different for DOBV-Aa and DOBV-Af strains. It is known from previous work of other authors that the exchange of as less as one amino acid in the glycoprotein of HTNV can significantly alter the virus pathogenicity (Ebihara *et al*., 2000; Isegawa *et al*., 1994). Practically nothing is known about the role of the viral RNA polymerase (product of the L segment) in host specificity and human pathogenicity.

However, at the current stage of knowledge it cannot be excluded that not only genetic differences between virus lineages in *A. agrarius* and *A. flavicollis* contribute to the different clinical outcome of DOBV-caused HFRS in Central/North-East/East Europe and South-East Europe, respectively. One of other possibilities to modulate the clinical outcome are certain genetic differences between human populations in Europe (see below).

4.5 Evolution of DOBV and the problem of hantavirus species definition

Our investigations gave new insights into the phylogeny of the DOBV virus group. It is surprising that related virus lineages (DOBV-Af and DOBV-Aa) are hosted by different rodent species (*A. flavicollis* and *A. agrarius*, respectively). Moreover, it has been known for a long time that *A. agrarius* in Asia (probably forming other subspecies different from the European subspecies) harbours a distinct virus species, HTNV.

To explain why two distantly related virus species, HTNV and DOBV, have been found in the same host species, *A. agrarius*, while two closely related DOBV lineages, DOBV-Af and DOBV-Aa, are hosted by two different rodent species, *A. flavicollis* and *A. agrarius*, a host switch has been suggested before (Plyusnin and Morzunov, 2001). Wang *et al*. (2000) and Nemirov *et al*. (2002) recently forwarded the idea that, probably, HTNV is the original hantavirus in the (Asian) *A. agrarius* and DOBV entered later this host species (rather in the European region) by host switch from *A. flavicollis* to *A. agrarius.*

In contrast to this, our results show the higher diversity of DOBV-Aa virus strains compared to the lower degree of divergency of the (so far determined) DOBV-Af strains, which might suggest an older evolutionary history of DOBV-Aa as compared to DOBV-Af. This would then lead to a scenario that DOBV-Af could have developed after a host switch from *A. agrarius* to *A. flavicollis*. The present amount of available data does not allow making a final decision between these possible scenarios. It should be mentioned that first experimental infections have shown that Saaremaa virus is able to infect both *A. agrarius* and *A. flavicollis* animals demonstrating the close relationship of the two virus-host systems (Klingstrom *et al*., 2002).

In addition, the question of host switch events playing a role in evolution of *Murinae*-associated hantaviruses was raised after the recent isolation of the HTNV-related hantavirus strain NC167 from *Niviventer confucianus* (Wang *et al*., 2000). Examples of potential host switch events have been also reported for hantaviruses associated with *Arvicolinae* (Vapalahti *et al*., 1999) and *Sigmodontinae* hosts (Nichol, 1999; Sanchez *et al*., 2001).

Since DOBV-Af and DOBV-Aa are stably associated with two different host species it has been proposed to consider them as different virus species and to take at that time the only available virus isolate of the DOBV-Aa group, Saa/160V, as the prototype of the new virus species, called Saaremaa (SAAV) (Plyusnin and Morzunov, 2001). According to the species demarcation criteria in the genus *Hantavirus*, as defined by the International Committee on Taxonomy of Viruses (Elliott *et al*., 1999), hantavirus species have to meet the following essentials:

(i) Species are found in a unique ecological niche, i.e., in a different primary rodent reservoir species or subspecies. This is the case for DOBV-Af versus DOBV-Aa. Our results presented here demonstrate that the investigated DOBV strains from *A. flavicollis* and *A. agrarius* in Slovakia are clearly different despite their co-existence at the same geographical place. On the other hand, the reassortment origin of Saaremaa virus indicates the possibility of genetic exchanges between members of the DOBV-Af and DOBV-Aa lineages. Obviously, the precondition of such events was the co-infection of the same host animal.

(ii) Species exhibit at least a 7 % difference in amino acid identity on comparison of the complete GPC and N protein sequences. We found aa differences in the deduced N protein sequences within the DOBV-Af and DOBV-Aa groups between 1.9 % (SK/Aa vs. AP/Af) and 3.3 % (Kur/44Aa vs. Esl/400Af and Slo/Af). Saa/160V shows a N protein sequence difference of 2.6 % when compared to the two DOBV-Af strains investigated. Since the Saa/160V carries a DOBV-Af-like S segment, it is not surprising that it exhibits lower similarities to the other DOBV-Aa strains than to the DOBV-Af strains. In the putative GPC the aa differences between the DOBV-Af and DOBV-Aa groups have been found in the range from 5.5% (Saa/160V vs. Slo/Af) to 6.6 % (SK/Aa vs. Slo/Af).

(iii) Species show at least a fourfold difference in two-way cross-neutralisation tests. By analysis of human serum samples from the Balkans (South-East Europe) and Estonia (North-East Europe) (Brus Sjolander *et al*., 2002) have shown that a majority of the sera exhibited this typical titer differences with preference for the local virus (DOBV-Slo/Af and Saa/160V, respectively). The authors conclude that DOBV-Af and Saaremaa virus define unique hantavirus serotypes. Obviously, the typical amino acid differences in the glycoproteins of the viruses (see above) are sufficient to result in the distinct serological behaviour.

(iv) Species do not naturally form reassortants with other species. However, this was highly probably the case for the Saa/160V virus (see above). The occurrence of such reassortment events between DOBV-Af and DOBV-Aa should be taken as one counter-argument against the idea that DOBV-Aa strains form a separate species. Moreover, even if one would like to consider the DOBV-Aa lineage as a unique virus species, the Saaremaa virus seems not to be the best representative of this suggested species because of its hybrid genome.

These observations highlight the importance of investigating carefully the relationship between proposed new viral species and/or lineages in determining which viral isolates represent true prototypes. In some cases, it will be possible to understand the specific and subspecific relationships among isolates only after examining a variety of members of a clade, including, whenever possible, those that occur sympatrically but in different host

species.

Based on our phylogenetic analysis, we like to suggest a distinction between at least three DOBV lineages. DOBV-Aa consists of strains originating from *A. agrarius* and is currently represented by strains from Central Europe and Russia including the Slovakia isolate. *A. flavicollis*-associated strains from Slovenia, Greece and Slovakia (Esl/400Af) represent the DOBV-Af lineage. The Saa/160V strain with its hybrid genome does not clearly belong to any of this two lineages and could be classified as an intermediate lineage (DOBV-Saa) (Figure 20). We believe that this classification should be kept within the DOBV species and no postulate of additional species is necessary. Particularly, grouping of in S and L segments non-monophyletic DOBV-Aa and DOBV-Saa lineages into one species and classifying DOBV-Af lineage as a second, distinct species as recently forwarded by Plyusnin *et al*. (2002, 2003), might not resemble the evolutionary history of these viruses.

4.6 Presence of distinct DOBV-Aa strains on single geographical locus

Our sequence analysis of six DOBV-Aa strains, detected in one trapping locality Rozhanovce, East Slovakia, revealed unusual results. Although detected in rodents captured in single locality, the partial S and M sequences, expected to be nearly identical, could be divided into two distinct groups showing the sequence diversity of up to 5.7%. In addition, phylogenetic analysis showed that the Rozhanovce-derived sequences do not cluster together in one monophyletic group.

Our hypothesis was that Rozhanovce might be situated on the border of the territories of two distinct *A. agrarius* subpopulations harbouring their own specific DOBV-Aa strains. In the molecular phylogenetic analysis of *Apodemus* mice based on 12S rRNA and D-loop (mitochondrial control region) mitochondrial markers, the obtained *A. agrarius* sequences appeared to be practically identical. However, an important question still remained to be answered. Do these results indicate that all individuals are members of an identical population or the selected markers are not suitable for a fine differentiation on population level?

Michaux *et al*. (2002) used 12S rRNA gene for studying the phylogeny of the genus *Apodemus*, but only on subgenus and species level. The suitability of 12S rRNA marker for these purposes was confirmed by our results showing that the sequences of *A. flavicollis* and *A. agrarius* were clearly distinct.

The mitochondrial control region was shown to be attractive to evolutionary biologists for fine scale comparative studies because it is believed to be one of the fastest evolving segments in the animal mitochondrial genome. For instance, the D-loop sequence was used to differentiate the subspecies *A. agrarius coreae* and *A. agrarius chejuensis.* The divergence in this marker within the subspecies was determined to be 2.98% and 1.86%, respectively (Koh *et al*., 2000). Nemirov *et al*. (2002) using D-loop, showed the distinctness of *A. agrarius* from Korea harbouring HTNV and *A. agrarius* from Europe. But also within the European *A. agrarius*, the sequences from Slovenia, Poland and Estonia were clearly distinguishable. On the other hand, Dekonenko *et al*. (2003) also used D-loop marker in the sequence analysis of the *Clethrionomys* voles from Finland, Sweden, and Western Siberia and, in line with our study, found extremely high degree of similarity within the species. In addition, similar results were obtained also with the mitochondrial cytochrome b gene, nuclear breast cancer susceptibility gene (BRCAI), and sex-determining region of chromosome Y (Sry-HMG) (Dekonenko *et al*., 2003).

From other phylogenetic markers, mitochondrial gene for cytochrome b (Martin *et al*., 2000; Serizawa *et al*., 2000; Barome *et al*., 1998), and nuclear genes IRBP (interphotoreceptor retinoid binding protein) (Michaux *et al*., 2002; Serizawa *et al*., 2000) and tspy (testis specific protein, Y chromosome-encoded) (Schubert *et al*., 2000) have been used in phylogenetic analyses of *Murinae* rodents, but resolved the relationships only on the species, subgenus or genus level.

Altogether, our study of *A. agrarius* in Slovakia did not confirm the presence of two distinct subpopulations, distinguishable at least in D-loop and 12S rRNA phylogenetic markers. The occurrence of two distinct strains of DOBV in a single locality might therefore have to be explained by another scenario than the presence of two distinct subpopulations of rodent hosts. Kratochvil (1962) concluded that the current distribution of *A. agrarius* in Central Europe is a result of multiple invasions and regressions and that this process is still ongoing. Therefore, we like to speculate that together with its host, DOBV strains of different origin could be rather recently “introduced” at least twice in the Eastern Slovakia region. Those distinct virus strains could now circulate in the current local *A. agrarius* population what would explain the presence of two distinct DOBV-Aa strains in phylogenetically uniform local rodent population.

It remained to be answered whether the data from Rozhanovce represent a rather exceptional or common picture of hantavirus biodiversity in natural foci and what consequences could that have for hantavirus epidemiology and ecology. It might be interesting to note that the prevalence of DOBV in rodents captured in Rozhanovce in 2001 was unusually high; 11 out of 42 (26.2%) mice were found sero- and RT PCR-positive.

4.7 A new DOBV strain isolated from *A. agrarius*

DOBV-Aa is an important HFRS pathogen in Central Europe; by serotyping and direct molecular proof dozens of patients with renal failure have been found to be infected with strains of this virus lineage (Klempa *et al.*, 2004; Sibold *et al.*, 2001; chapters 3.4, 3.5). However, all current knowledge about the genetics and molecular phylogeny of DOBV-Aa strains was generated on the basis of nucleic acid isolation from *A. agrarius*- or human-derived specimens and subsequent PCR amplification and nucleotide sequence analysis. Here we describe the isolation of an indigenous DOBV-Aa virus strain which can be taken as the representative of the DOBV-Aa lineage within the DOBV species.

Our sequence and phylogenetic analysis showed that the DOBV-Aa virus isolate, named Slovakia or SK/Aa in brief, is genetically closely related to the other Central European DOBV-Aa sequences. When considering the S and L segment phylogenetic trees of DOBV strains, SK/Aa is the only viable virus strain within the whole DOBV-Aa genetic lineage.

Hantavirus isolation is a tedious, time-consuming process, which is only rarely successful. When original DOBV strain from Slovenia (Slo/Af) was isolated, only one out of 13 isolation attempts was successful (Avsic-Zupanc *et al*., 1992). However, an isolation protocol used for isolation of Saa/160V, including three weeks passaging intervals and adding of fresh uninfected cells to passaged cells, seems to be very efficient. Nemirov at al. (1999) could isolate virus from three out of three tissue samples. We also reached 100% efficiency, when both our isolation attempts were positive. Besides the protocol, critical points might be the tissue homogenisation step and the viral load in naturally infected rodent tissues. We selected samples very strongly reacting in screening tests (ELISA, RT-PCR) and the tissues were triturated very thoroughly using FastPrep Instrument (BIO 101, USA). Usually the lung tissues of rodents are used for isolation attempts. Our results suggest that liver tissues can be used as well. This could be an advantage because the amount of lung tissue is very limited and, moreover, lung tissues are usually used also for initial RT-PCR screening of animals. Vapalahti *et al*. (1996) used lung tissues and a pool of liver, kidney and spleen tissues in parallel during their TULV isolation attempts, though only lung tissues revealed a positive result.

The SK/Aa isolate was grown in Vero E6 cells. Adaptation of a field virus to cell culture may be accompanied by the accumulation of mutations in the viral genome. However, when we compared the original sequence from the *A. agrarius* specimen used for virus isolation (Esl/34Aa) with the nucleotide sequence of the complete S segments of the virus isolate SK/Aa, we found no differences in the non-coding regions and only one putative amino acid exchange in the N protein occurred. This exchange of Ala to Thr on aa position 43 can be

considered as conservative according to the criteria of Dayhoff and co-workers (1978). Both aa residues can be also alternatively found in the N protein sequences of other hantavirus species, no matter whether the respective strains were cell-adapted or „wild“ strains (data not shown). This let us conclude that our virus isolate at least in the S segment represents the natural genetic make-up of the „wild“ virus.

Similar analyses were recently undertaken with PUUV strain Kazan (Lundkvist *et al*., 1997b; Nemirov *et al*., 2003a); the only amino acid substitution was found in the L protein, Ser versus Phe at position 2053. In the entire S segment sequences, single mutations in both 5’ and 3’ NCRs have been observed which correlated with a different infectivity of the viruses to bank voles, but no differences could be found in S segment coding region and entire M segment (Lundkvist *et al*., 1997b).

In other studies, the complete S segment sequence recovered directly from the lung tissue sample of *A. flavicollis* was found to be identical to that of the subsequent Vero E6 cell culture isolate Dobrava/Ano-Poroia (Nemirov *et al*., 2003b). Chizhikov *et al*. (1995) compared the non-translated regions of the S and M segments of SNV RNAs amplified from the tissues of the original trapped and experimentally infected *Peromyscus maniculatus* rodents with virus harvested from the fifth passage in Vero E6 cells and found no nucleotide sequence differences among these samples suggesting that no genetic selection or adaptation is taking place during growth of the virus in the experimentally infected *P. maniculatus* as well as during the five passages in Vero E6 cells.

Since the amplification of hantaviral nucleic acid from patient's material is difficult and rarely successful, FRNT is the only useful method for fine typing of human hantavirus infections in HFRS diagnostics (Krüger *et al*., 2001). The validity of the approach to serotype neutralising antibodies in the patient’s serum by FRNT mainly depends on the availability of that virus in the assay which is nearly related to and therefore representative for the naturally infecting virus strain. Accordingly, the SK/Aa strain is the virus of choice to test sera from Central European HFRS patients in FRNT. Here we have investigated convalescent sera from 9 HFRS patients from Germany and Slovakia originally diagnosed as DOBV-positive on the basis of the neutralising ability of their sera towards the Slo/Af prototype strain (Sibold *et al*., 2001; chapter 3.6). When comparing the neutralisation endpoint titers against SK/Aa versus Slo/Af of these patients’ sera, we found an at least fourfold higher reciprocal end-point titer towards SK/Aa in 5/9 sera. Two out of nine sera exhibited equal neutralisation activities towards SK/Aa and Slo/Af, and the remaining 2/9 sera neutralised the Slo/Af virus strain significantly better than the SK/Aa strain (Table 18).

Since in our work (using sera of HFRS patients) as well as in seroprevalence studies in Estonia (Brus Sjölander *et al*., 2002; Golovljova *et al*., 2002), Latvia (Lundkvist *et al*., 2002a)

and most of all in Lithuania (S. Sandmann, H. Meisel, A. Razanskiene, A. Wolbert, B. Pohl, D.H. Krüger, K. Sasnauskas, and R. Ulrich, submitted for publication) a noticeable number of sera reacted equally well with Slo/Af on the one hand and SK/Aa (or Saa/160V) on the other, it is reasonable to speculate that the envelope glycoproteins (encoded by the viral M segments and responsible for the reaction with the neutralising antibodies) of DOBV-Af and DOBV-Aa are not diverse enough to account for clear differences in the patterns of neutralising antibodies. Alternatively, when one takes an at least fourfold higher end-point titer as evidence for the infection of the patient by the respective virus strain, our FRNT data could be interpreted in the following way. The majority of patients (5/9) was infected by DOBV strains nearly related to our SK/Aa isolate, for 2/9 patients no conclusion can be made, and further 2/9 patients could have been infected by DOBV-Af-like virus strains. Interestingly, both sera exhibiting better neutralisation of the Slo/Af strain were taken from patients from Slovakia, a country in Central Europe not far from South-East Europe where DOBV-Aa and DOBV-Af were demonstrated to be sympatrically present (Sibold *et al*., 2001). Our data provide a first hint that in this geographical region not only DOBV-Aa but also DOBV-Af strains could be etiological agents of DOBV-associated HFRS cases (see below).

In summary, the first Central European DOBV strain has been isolated from an *A. agrarius* rodent. The availability of this virus strain will allow additional studies to answer such interesting questions as about the antigenic properties of DOBV lineages, their differential virulence potential, or the role of genetic reassortment and host-dependent virus evolution.

4.8 HFRS in West Slovakia associated with DOBV-Af infection

We have described two DOBV-associated HFRS cases from West Slovakia. A few DOBV cases can be detected in this region every year. Interestingly, only *A. flavicollis* is present in this part of the country suggesting that DOBV-Af is an etiologic agent of DOBV cases in this region. Human infections caused by DOBV-Af have been yet reported only from South-East Europe, where the HFRS cases caused by DOBV infection were found to be rather clinically moderate or severe. Nevertheless, the severity of diseases and fatality rate in West Slovakia are not similar to those in South-East Europe but rather resemble the situation in rest of Central Europe. The only two HFRS fatal cases from western part of the country were documented in 1958 and 1959 (Dornetzhuber *et al*., 1960). It was therefore interesting to clinically describe these infections and to investigate their association with DOBV-Af.

The association with DOBV-Af was confirmed when sera of both patients showed fourfold higher reciprocal titer against Slo/Af than against SK/Aa. This finding represents in

fact the first, although only indirect evidence that DOBV-Af is an etiologic agent of DOBV cases in Central Europe.

The estimation of HFRS severity according to criteria of Lee and van der Groen (1989) showed interesting results. In both of the cases most of the parameters suggested only mild diseases. In contrast, two criteria (max. BUN and days of proteinuria) of patient A corresponded to severe disease. Both parameters are related with the acute renal failure of patient A. It is questionable, how relevant these criteria, originating from clinical data of HTNV infections in Korea, for DOBV infections are. Nevertheless, two described cases showed similar clinical course as reported previously for DOBV-associated HFRS cases from Central Europe (Sibold *et al*., 2001).

4.9 First direct evidence that DOBV causes HFRS in Central Europe

For the first time, DOBV genetic material could be directly detected in a specimen from a patient in Central Europe. Generally, most of the hantavirus sequences available in GenBank were obtained from rodent tissue samples or virus isolates and sequences obtained from patient material are rather exceptional. It is mostly due to a fact that patient blood samples are very often taken too late. Hantaviral RNA is usually detectable only within the first days after onset of disease and not even in all patients (Vapalahti *et al*., 2001; Horling *et al*., 1995; Plyusnin *et al*., 1997a; Plyusnin *et al*., 1999a). The second critical point might be the handling and storing of clinical material. Our first positive outcome suggests that using of RNA-preserving buffer is necessary to keep detectable level of viral RNA, if the samples could not be immediately stored at –70°C. In our case, storing the blood sample in AVL Buffer (Qiagene Viral RNA Kit) at –20°C allowed us to detect and amplify hantaviral RNA and a partial S segment nucleotide sequence, designated H169, could be obtained.

Sequence analysis of H169 clearly revealed that this strain from a HFRS patient in Central Europe belongs to the DOBV species. The putative amino acid sequence encoded by the analysed genome fragment showed a 99.4 % identity with the DOBV-Aa virus sequences from Slovakia, Central Europe. The molecular phylogenetic analysis exhibited clustering of the H169 strain with sequences of the DOBV-Aa lineage suggesting that a DOBV strain originating from *A. agrarius* was responsible for the described HFRS case. Interestingly, DOBV-Aa strains from East Slovakia have been shown to have the highest sequence identity to H169, although in phylogenetic tree they occupy the most distal and ancestral positions, respectively.

Interestingly, the clinical course of this infection (patient H169) was quite similar to the more severe DOBV-Af cases reported from West Slovakia. Despite of acute renal failure, according to Lee and van der Groen (1989) many parameters suggested only mild disease. It seems to be characteristic for DOBV infections in Central Europe that only laboratory parameters related to renal failure are comparable to severe HTNV infections from Asia (Lee and van der Groen, 1989). Other markers, particularly those corresponding to febrile phase, usually indicate only mild disease, when haemorrhages, fever over 39°C or leucocytosis are rather exceptional. It might be concluded that DOBV in Central Europe causes infections with acute renal failure, however, without such severe hemorrhagic complications as observed for HTNV or eventually for DOBV in Balkans.

Altogether, for the first time a direct evidence that DOBV causes HFRS in Central Europe was obtained. The sequence originating from blood sample of an HFRS patient from North-East Germany is clearly distinct from patient-associated DOBV sequences from Greece and is closely related to DOBV strains found in *A. agrarius* mice.

4.10 Questioning the hypothesis about different pathogenicity of DOBV-Af and DOBV-Aa towards humans

Recently, several authors forwarded the hypothesis that DOBV-Af and DOBV-Aa harbour different pathogenicity towards humans. This idea is mostly based on fact that many severe HFRS cases have been reported from the Balkans, where DOBV-Af is believed to be dominant (Avsic-Zupanc *et al*., 1999; Papa *et al*., 1998). On the other hand, no fatalities and only mild DOBV-associated diseases occur in Central and North-East Europe, where DOBV-Aa is supposed to prevail (Sibold *et al*., 2001; Brus Sjolander *et al*., 2002; Plyusnin, 2002). However, the comparison of our three DOBV clinical cases (chapters 3.5 and 3.6) suggests that there is no obvious difference in severity of DOBV infections in Central Europe.

The species range of *A. flavicollis* covers almost the whole Europe (Montgomery, 1999) and it seems to be unlikely that the distribution of DOBV-Af is restricted only to Balkans. The detection of DOBV-Af in Slovakia (Sibold *et al*., 2001; Klempa *et al*., 2003b; chapter 3.1) and the putative DOBV-Af-associated clinical cases from West Slovakia (chapter 3.6) are in line with this assumption. Nevertheless, a high severity and fatality rate of HFRS is reported only from Balkans. It might be concluded that not DOBV-Af generally, but only local strains, circulating in Balkans harbour higher pathogenicity towards humans.

Moreover, these are not necessarily the properties of the virus which are responsible

for the different clinical outcome of the disease. Genetic variation in immune related genes of human populations is well known to be associated with interesting immunological phenotypes including susceptibility to diseases. Four such polymorphic regions are well-established candidate regions for disease susceptibility; T cell receptor loci, the killer Ig-like receptor loci, the immunoglobulin heavy chain region, and the major histocompatibility complex (Geraghty, 2002). The recent epidemic of severe acute respiratory syndrome (SARS) in Taiwan is a recent example how the genetic differences among human population can influence the outcome of infectious diseases. Up until recently, no probable SARS patients were reported among Taiwan indigenous people who are genetically distinct from the Taiwanese general population, do not show the human leukocyte antigen (HLA) B*4601 allele and have high frequency of HLA-B*1301 allele (Lin *et al*., 2003).

For other examples, one even does not have to go far from DOBV. It was shown that HLA haplotypes can influence the clinical course of hantavirus infection, as shown for PUUV infection. Mäkelä *et al*. (2002) showed that the HLA-B8-DR3 haplotype is an important contributor to the course of *Nephropathia epidemica*. In addition, patients with the most severe course of the disease had a very high frequency of HLA B8, C4A*Q0, and DRB1*0301 alleles (Mustonen *et al*., 1996). It has also been shown that non-carriage of IL-1RA allele 2 and IL-1Beta (-511) allele 2 may contribute to susceptibility to PUUV infection. In contrast, HLA B27 seems to be associated with a benign clinical course of PUUV infections. It is also of interest that there are apparently similar associations with HIV disease; the fast progression is associated with B8 DRB1*0301 whereas B27 is associated with milder forms (see Mustonen *et al*., 1998).

Therefore, certain genetic differences between human populations in Europe could contribute to the different clinical outcome of DOBV-caused HFRS in Balkans in contrast to the rest of Europe. In recent population genetic studies, some differences between Balkans and other South and Middle European populations and even within the Balkan population have been found in serum proteins (Scheil *et al*., 2001), DNA-Short Tandem Repeat (STR) analysis (Huckenbeck *et al*., 2001), and allele frequencies of alpha-1-antitrypsin gene (Scheil *et al*., 2002). It cannot be excluded that Balkans human population differs also in some markers associated with hantavirus infection susceptibility.

Altogether, the presence of severe HFRS cases in the Balkans and only mild DOBV-associated diseases in Central and North-East Europe might be explained by different pathogenicity of DOBV-Af and DOBV-Aa towards humans, but also by higher virulence of local Balkan DOBV-Af strains or some immunogenetic differences in human populations in Europe. With the current state of knowledge, any of these scenarios might be valid. The characterisation of reasonable numbers of DOBV-Af cases outside Balkans and eventually

also DOBV-Aa cases from Balkans, as well as population genetic studies are needed to clarify this important subject.

4.11 Does pathogenic TULV circulate in North-East Germany?

So far, TULV was considered to be probably non-pathogenic for humans. One singular case of (anamnestic) human infection could be found by serological evidence in a healthy blood donor indicating that TULV (or a TULV-like virus) might be able to infect humans (Vapalahti *et al*., 1996). Recently the first case of an acute TULV infection of a patient with fever, paronychia and exanthema but without renal or pulmonary affection has been reported. However, the disease occurred after the patient was bitten by a wild rodent, indicating an unusual route of hantavirus transmission. Similarly to our case, several bouts of fever occurred during the course of infection. Interestingly, the rodent involved in this case was later described by the patient (12-year-old boy), his mother and sister, but the description was not compatible with the characteristics of *M. arvalis,* the natural carrier of TULV (Schultze *et al*., 2002).

In our case some typical signs of HFRS (e.g. proteinuria, polyuria) could be observed. Moreover, no unusual route of infection could be suggested. On the other hand, two phases of disease and pneumonia were observed, signs not common for HFRS. In contrast to the previous report (Schultze *et al*., 2002), the hantavirus specific IgM antibodies could not be detected by IgM-specific ELISA. However, such disappearance of detectable IgM within four weeks after onset of disease has also been described for other cases of hantavirus infections (Elgh *et al*., 1998; Kallio-Kokko *et al*., 1998). An acute hantavirus infection was shown by the increase of hantavirus antibody titers against PUUV antigen determined by IFA from 1:128 (day 2 after onset), through 1:256 (day 12) to 1:3,200 (day 38). The FRNT is the only choice for precise identification of hantavirus infection if the viral genetic material can not be detected. The at least fourfold higher reciprocal titer against TULV when compared with the other viruses, indicated that TULV was the virus responsible for the infection of the patient. The FRNT titer against TULV was relatively low, but the same titer (160) was obtained also in the previous TULV case (Schultze *et al*., 2002). Nevertheless, it cannot be completely excluded that a TULV-related, albeit unidentified virus not present in the FRNT virus collection caused the infection.

It was thus important to show that TULV is endemic in this region of Germany. TULV strains were detected in *M. arvalis* animals trapped at places only a few kilometres distant from the home village of the patient. On ML phylogenetic tree, these strains clustered with

strains from Poland and represent a new, well-supported third sublineage within the TULV species. It might be possible that the pathogenicity towards humans is unique for this newly characterised sublineage.

Up to now, two branches (sublineages) of TULV had been defined represented by strains from Russia on one hand and strains from Czech Republic and west Slovakia on the other hand . The sequences of TULV strains from East Slovakia (Y13979 and Y13980) have been considered to be recombinants between these two major sublineages (Sibold *et al*., 1999a). Therefore we excluded them from the phylogenetic analysis of complete S segment sequences. Recently, TULV was detected in Belgium and these strains together with sequences from Switzerland and south Germany were postulated to represent an additional sublineage (Heyman *et al*., 2002). However, too short partial S segment sequences (304 nt) were used for phylogenetic analysis and the overall bootstrap support for calculated tree was not plausible.

Altogether, our data let us conclude that TULV is circulating in North-East Germany and that the virus is a causative agent of renal and pulmonary affection in humans. In addition to PUUV and DOBV, TULV should be considered as the third Central European hantavirus being pathogenic for humans.

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APPENDIX

Abbreviations

aa	amino acid(s)
ANDV	Andes virus
BSA	bovine serum albumin
BUN	blood urea nitrogen
DOBV	Dobrava virus
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FFU	focus forming unit
FITC	fluorescein isothiocyanate
FRNT	focus reduction neutralisation test
GPC	glycoprotein precursor
HBSS	Hanks' balanced salt solution
HCPS	Hantavirus cardiopulmonary syndrome
HFRS	Hemorrhagic fever with renal syndrome
HLA	human leukocyte antigen
HTNV	Hantaan hantavirus
ICTV	International Committee on Taxonomy of Viruses
IFA	immunofluorescence assay
KHAV	Khabarovsk virus
MAb	monoclonal antibody
MHC	major histocompatibility complex
ML	Maximum likelihood
N	nucleocapsid protein
NCR	non-coding region
nt	nucleotide(s)
ORF	open reading frame
PBS	phosphate-buffered saline
PUUV	Puumala hantavirus
RT-PCR	reverse transcription – polymerase chain reaction
SEOV	Seoul virus
SNV	Sin Nombre virus
TOPV	Topografov virus
TULV	Tula hantavirus

Acknowledgement

First of all, I would like to thank to Professor Detlev H. Krüger for giving me the opportunity to come to Berlin and work in his laboratory. I am very grateful for his support and supervision of my work. He has always been there with help or advice and ready for fruitful discussion.

I would like to thank to Dr. Helga Meisel and all members of her working group , Anne Endres, Stefanie Märschenz, Astrid Schories, and Heike Lerch, for creating enjoyable atmosphere and being always ready to help me. Brita Auste is especially acknowledged for her essential help and excellent technical skills. I am also thankful also to all the other colleagues from the Institute of Virology, Charité Berlin, who helped me during my work. Beate Ziaja is acknowledged for introducing me to the work under biosafety level 3 containment conditions.

I would like to thank Dr. Milan Labuda for his continuous support and stimulating discussions through e-mail and to his co-workers from the Department of Virus Ecology, Slovak Academy of Sciences, Bratislava for their help in collecting rodent samples. The contribution of our collaborating partners, Morten Schütt, Brian Hjelle, Maria Stankovicova, and Michal Stanko is greatly appreciated as well.

My special thanks goes to Heiko A. Schmidt who helped me a lot during my “baby steps” in phylogenetic analysis.

Last but not least, I am extremely grateful to my girlfriend Lenka, my parents, and brother Rasto for their constant love and support in many difficult situations during my stay in Berlin. I am also very thankful to all my new German friends who helped me to find a second home in Berlin.

I would like to dedicate this thesis to Dr. Oto Kozuch, the supervisor of my Diplom thesis who died shortly before I started this work.

List of Publications

Klempa, B., Schmidt, H.A., Ulrich, R., Kaluz, S., Labuda, M., Meisel, H., Hjelle, B., Krüger, D.H. (2003). Genetic interaction between distinct Dobrava hantavirus subtypes of *Apodemus agrarius* and *A. flavicollis*: Implications for host-specific evolution and adaptation in nature. *J Virol*, 77, 804-809.

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Klempa, B., Auste, B., Schütt, M., Ulrich, R., Labuda, M., Meisel, H., Krüger, D.H.: First direct evidence that Dobrava virus causes hemorrhagic fever with renal syndrome in Central Europe. Annual Meeting of the German Society of Virology (GfV), Berlin, Germany. March 2003 – oral presentation

Klempa, B., Meisel, H., Räth, S., Ulrich, R., Krüger; D.H.: First evidence that Tula hantavirus is associated with the occurence of renal and pulmonary syndrome. Annual Meeting of the German Society of Virology (GfV), Berlin, Germany. March 2003 – poster

Klempa, B., Auste, B., Schütt, M., Ulrich, R., Meisel, H., Krüger, D.H.: First viral nucleotide sequence amplified from a patient infected by Dobrava virus in Central Europe. Twelfth International Conference on Negative Strand Viruses, Pisa, Italy. June 2003 – poster

Klempa, B., Schmidt, H.A., Ulrich, R., Schütt, M., Labuda, M., Hjelle, B., Meisel, H., Krüger, D.H.: Sympatric existence and genetic interaction of Dobrava hantavirus lineages carried by different host species. Twelfth International Conference on Negative Strand Viruses, Pisa, Italy. June 2003 – poster

Klempa, B., Krüger, D.H.: Phylogenetic analysis of DOBV strains from Central Europe.

Ninth International Workshop on Virus Evolution and Molecular Epidemiology, Stanford, USA, August 2003- oral presentation

Klempa, B., Stanko, M., Labuda, M., Ulrich, R., Meisel, H., Krüger, D.H.: Dobrava hantavirus as an emerging virus in Central Europe: Characterisation of the first virus isolate. Annual Meeting of the German Society of Virology (GfV), Tuebingen, Germany. March 2004– oral presentation

Klempa, B., Krüger, D.H., Schmidt, H.A.: Molecular evolution of Murinae-associated hantaviruses. Sixth International Conference on HFRS, HPS and Hantaviruses, Seoul, Korea. June 2004 – oral presentation

Krüger, D.H., **Klempa, B.**, Ulrich, R., Schönrich, G., Labuda, M., Meisel, H.: Distribution of different pathogenic hantaviruses in Central Europe. Sixth International Conference on HFRS, HPS and Hantaviruses, Seoul, Korea. June 2004 – oral presentation

Meisel, H., **Klempa, B.**, Ulrich, R., Haring, B., Schütt, M., Schwarz, A., von Müller, L., Krüger, D.H.: Hantavirus infections in Germany: Hemorrhagic fever with renal syndrome cases due to Puumala, Dobrava, and Tula virus infections. Sixth International Conference on HFRS, HPS and Hantaviruses, Seoul, Korea. June 2004 – oral presentation

Klempa, B., Stanko, M., Labuda, M., Ulrich, R., Meisel, H., Krüger, D.H.: Dobrava hantavirus in Central Europe: Characterisation of the virus isolate from striped field mouse (*Apodemus agrarius*) captured in Slovakia. Sixth International Conference on HFRS, HPS and Hantaviruses, Seoul, Korea. June 2004 – poster

Schmidt, J., Jandrig, B., Khoprasert, Y., **Klempa, B.**, Yoshimatsu, K., Arikawa, J., Meisel, H., Niedrig, M., Krüger, D.H., Jäkel, T., Ulrich, R.: Detection of hantavirus-reactive sera in wild-trapped rodents from Thailand by newly developed immunological assays based on yeast-expressed Seoul virus nucleocapsid protein. Sixth International Conference on HFRS, HPS and Hantaviruses, Seoul, Korea. June 2004 – poster

Selbständigkeitserklärung

Hiermit versichere ich, daß ich die vorliegende Dissertation „**Dobrava and Tula hantaviruses from Central Europe: molecular evolution and pathogenic relevance**“ selbst verfaßt habe und auch in Teilen keine fremden Arbeiten darin enthalten sind. Teile dieser Arbeit waren bzw. werden Gegenstand eigener Publikationen. Die verwendeten Hilfsmittel und Literatur habe ich vollständig angegeben.

Berlin, Juli 2004

Boris Klempa